THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME VII JULY, 1931 NUMBER 4

EXPERIMENTAL HEPATIC PIGMENTATION AND CIRRHOSIS*

I. DOES COPPER POISONING PRODUCE PIGMENTATION AND CIRRHOSIS OF THE LIVER?

ERNEST M. HALL, M.D., AND EATON M. MACKAY, M.D.

(From the Department of Pathology of the University of Southern California, School of Medicine, Los Angeles, and The Scripps Metabolic Clinic, La Jolla, California)

It is now ten years since Mallory, Parker and Nye[1] demonstrated the presence of pigmentation and cirrhosis of the liver in rabbits following the administration of copper salts in the feed. These authors called attention to the close similarity of the experimental lesions thus produced to those of hemochromatosis in man. Hall and Butt,[2] in 1928, repeated and extended the work of Mallory and his associates, obtaining pigmentation of the liver with early cirrhosis by feeding copper acetate and by injecting a weak solution of the same salt subcutaneously. These investigators found, on chemical analyses of the rabbits' livers, the presence of large quantities of copper in amounts roughly proportional to the various degrees of pigmentation.

In January, 1929, Flinn and VonGlahn [3] published the results of an investigation in which some of the earlier experiments of Mallory and his associates [1] were repeated and other experiments of their own added. These authors concluded that neither copper nor its compounds cause the deposition of pigment in the livers of rabbits, guinea pigs or rats, and neither do they produce cirrhosis. They also stated that a diet consisting exclusively of carrots will produce pigmentation of the liver in rabbits "in every way identical with that ascribed to copper." In an editorial[4] in *The Journal of the*

* Received for publication April 29, 1931.

American Medical Association of February 2, 1929, the editor cites the results of Flinn and VonGlahn to illustrate his remarks concerning the difficulties of animal experimentation. Admitting this report to be a "categorical denial" of the results of Mallory, he accepts, as proved, several statements which the authors hope to show are incorrect: first, the general assertion that copper and its compounds do not cause the deposition of pigment in the livers of laboratory animals; second, that the amount of copper in the tissues in experimental hemochromatosis is about the same as that in healthy tissues; third, that pigment cirrhosis can be produced in rabbits with sodium acetate; and finally, that experimental pigment cirrhosis in rabbits is probably caused by a diet top-heavy with carrots.

Polson,[5] working in England, has since published results which agree in general with the conclusions of Flinn and VonGlahn. He reported that copper acetate failed to produce a higher incidence of pigment cirrhosis in the liver than is found in normal controls when all of the rabbits are fed on cabbage, oats, bran and thirds. In a series of control rabbits fed on a diet containing mangel-wurzels and turnips, there was an accumulation of "hemofuscin in the livers of 88 per cent of the animals."

During the past two years German investigators have been very active in studying the "copper problem." Herkel,[6] in May, 1930, published a general review dealing with the biology and pathology of copper poisoning, including some experimental work. He has made a very complete review of the literature on experimental copper poisoning and the reader is referred to this article for a full account of the literature.

According to Herkel some of the earlier experiments were conducted by German and French workers about the middle of the 19th century. Among the earlier German investigators Ellenberger and Hofmeister,[7] 1883, obtained marked degenerative changes and deposition of pigment in the livers of sheep due to feeding copper sulphate. Filehne,[8] 1896, fed rabbits copper sodium-acid-tartrate and obtained fatty degeneration of the liver, connective tissue and bile-duct proliferation, together with a flaky pigmentation of the liver cells. A dog fed for two months on copper stearate gave a similar picture in the liver. In the same year Baum and Seeliger [9] obtained very definite changes in the livers of dogs, sheep, goats and cats (22 animals in all) following the feeding of cuprohemol, copper sulphate,

copper acetate and copper oleate for periods of time up to one year. Besides the changes in the liver described by Filehne [8] these authors speak of the presence of an iron-containing pigment, hemosiderin, and an iron-free pigment which they call hematoidin. The pigment was found especially in the parenchyma cells. Similar changes are described as occurring in the kidneys. On chemical analysis of the livers a copper content was found as high as 0.2 per cent (200 mg. copper for 100 gm. liver substance).

More than a dozen of the earlier investigators cited by Herkel [6] obtained negative results in their copper-feeding experiments. Most of these studies, however, were not so carefully conducted or so well controlled as were those just cited. In several cases the duration of the experiments, or the dosage of copper employed, was not sufficient to produce pigmentation or cirrhosis.

Herkel's experiments are as follows: twelve rabbits were given daily 25 to 50 mg. of different copper salts (sodium tartrate, stearate and chloride) over periods of 8 to 154 days. Another group of five rabbits was given various combinations of the above copper salts combined with iron salts. These animals when killed showed in some cases a dark greenish brown color of the liver, otherwise they were normal. Histological studies showed rich deposits of round or granular pigment in the liver cells such as may be observed in the rabbit's liver under physiological conditions. The pigment was diffusely distributed in the liver cells. Only one animal showed connective tissue proliferation and this was not typical of cirrhosis. In general, their results agree with those of Flinn and VonGlahn and of Polson.

Eight rats were fed various copper salts (25 to 50 mg. daily for 42 to 112 days) as in the previous experiment, and eleven others were fed a combination of copper and zinc salts (25 mg. of each daily for 70 to 77 days). The results of the rat experiments were negative.

Chemical analyses of the rabbit and rat livers showed fairly large amounts of copper stored in the liver tissues. Quantities of copper up to 0.1 per cent failed to produce cirrhosis.

In a report from Aschoff's laboratory by Oshima and Siebert,[10] 1930, the question of experimental copper poisoning is reviewed and the results of their experiments recorded. This work was undertaken, according to Schönheimer,[11] because of the discovery of ten to twelve cases of hemochromatosis in Freiburg within the period of about a year, and further on account of the fact that the extensive

grape vineyards of that region are treated with copper salts to prevent the growth of mildew and other fungi. Nine animals were given 200 mg. of copper acetate daily by mouth, while three others were given similar quantities of zinc acetate. One animal of the copper group died early of infection, the others were fed for from 61 to 249 days. The copper content of the livers varied from 52 to 170 mg. per kilogram of liver tissue, an average of 85 mg. Six normal rabbit livers were analyzed and yielded 6 mg. per kilogram of liver. There was considerable pigment deposited in the liver cells but in only two cases was pigment found in the Kupffer cells. The pigment did not stain selectively with fuchsin. There was slight increase in connective tissue but no true cirrhosis. The zinc-fed animals also developed moderate pigmentation of the liver cells. These authors failed to find the complex picture of early hemochromatosis comparable with that presented by Mallory, but on the other hand, they would not agree with the conclusions of Flinn and VonGlahn. They believe that copper alone is not responsible for the changes in the liver, but that some unknown factor plays a rôle.

Adrianoff and Ansbacher,[12] 1930, reported that cirrhosis developed in three out of four rats that were given copper. The presence or absence of pigmentation was not mentioned.

It is obvious, from a consideration of the wide divergence of results obtained by these various workers, that a reinvestigation under very exact and well controlled conditions of the whole question of copper poisoning in relation to hepatic pigmentation and cirrhosis, is desirable. Copper administered in sufficient quantities either does, or does not, produce pigmentation and cirrhosis of the liver in rabbits. The present investigation was undertaken for the purpose of answering this question. Whether or not the feeding of carrots, cabbage and other substances produces pigmentation, will be considered in another study.

METHODS

In view of the conflicting results that have been obtained in the studies just cited, it was realized that exact methods and carefully controlled experiments would be necessary, if results of value were to be obtained. Rabbits of good stock were used throughout, since these animals are more sensitive to hepatic poisons than are rats or guinea pigs. The rabbits used were kept in individual, large,

roomy, well protected outdoor hutches, with twelve square feet of floor space, placed on a hillside with a southeast exposure. The temperature range of 55° to 70° F was not exceeded at any time.

An attempt was made to keep the conditions of the copper-fed group and the control group exactly the same, except that one group received copper in the form of normal copper acetate added to their food. This desirable state of affairs was nearly attained. The one difference, as we shall see, rested in the loss of appetite of the copper-fed rabbits and their failure to eat as much food as did the controls.

All of the rabbits were placed on a special control diet when they were exactly 85 days of age. This diet consisted of a mixture of 75 parts of whole ground alfalfa and 25 parts of whole ground barley. The control rabbits continued to receive this diet until the end of the experiment, while the copper group commenced to receive the copper diet when exactly 90 days of age. The copper diet was the same as the control diet, except that it contained 2 mg. of copper per gram of food in the form of normal copper acetate. The copper-containing food was prepared by the addition of 10 cc. of 63 per cent copper acetate solution to each kilo of the control diet. Actually the copper solution was added to a small portion of the food, which was then dried at 50° C, after which it was intimately mixed with the remainder. These diets, together with tap water, were allowed the respective groups *ad libitum*. For every rabbit which received the copper diet, a *litter-mate of the same sex* was fed the similar but non-copper-containing control diet. This is generally looked upon as the optimal method of control in animal feeding experiments.

The experiments were not continued for any definite length of time. Some of the copper-fed rabbits died and their controls were killed after the experiment had continued for 21 to 60 days. All but five were left on the copper diet until they succumbed, presumably from copper poisoning. It is reasonable to suppose that these animals died of copper poisoning because not a single control rabbit died before being sacrificed at the time of death of its copper-fed litter-mate. All of the control animals were in excellent condition when killed. The food intake was measured daily. Although there was some unavoidable scattering, the diets were both very light, and we believe that the food intake figures are reasonably accurate. For present purposes the copper intake of the *control* rabbits was so small that it is negligible, while the copper-fed rabbits received 2 mg.

of copper with each gram of food. The rabbits were weighed at five-day intervals.

When a copper-fed rabbit was killed or died, its litter-mate control was also sacrificed and the carcass carefully examined. Since the intestinal contents of the rabbit constitute a variable but considerable portion of the body weight, the weight of the entire gastro-intestinal tract and its contents was subtracted from the gross body weight. The "net" body weight so obtained was used for comparison. The liver in each case was carefully removed, and if both the copper-fed and the control animals had been killed, each was drained of gross blood. If the copper-fed rabbit had been dead for some hours, no attempt was made to free the control liver of blood. The liver was weighed and representative pieces of tissue taken for histological examination. The remainder of the organ was again weighed, dried at 105° C to constant weight, and preserved for biochemical analyses. Many of the animals showed some coccidial infestation. If one of a pair was infested, the litter-mate was usually also infested. In any case this factor appeared to have little influence on the final results. All the other viscera besides the liver were examined grossly, and histological studies were made in a few cases. No changes of consequence were found outside of the liver.

The amount of copper in the livers was determined by the micromethod of Elvehjem and Lindow.[13] This method was checked in a number of instances by the standard macromethod.

Blocks of liver tissue from each animal were fixed in 95 per cent alcohol and in formalin. In a few cases blocks from other organs were also fixed in these solutions. The alcohol-fixed material was kept in reserve. Sections were stained with hematoxylin and eosin; also with potassium ferrocyanide and hydrochloric acid followed by a 0.5 per cent solution of basic fuchsin in 50 per cent alcohol. This stain causes hemosiderin granules to assume a dark blue color (Berlin blue reaction for iron), while the basic fuchsin stains hemofuscin deep red in either the presence or absence of hemosiderin. Another method, recommended by Mallory,[1] was found very useful, especially if photomicrographs showing the hemofuscin were to be made. This method consists in staining the sections in alum hematoxylin, followed by a mixture of 1 part of strong yellow ammonium sulphid to 3 parts of 95 per cent alcohol for 1 to 2 hours. The latter operation should be carried out in a glass staining dish with a tightly fitting

cover to prevent loss of the ammonia. After thorough washing in water, sections are stained in 0.5 per cent basic fuchsin as described above for 20 minutes. Destain in 95 per cent alcohol and dehydrate, running through absolute alcohol, xylol, and mounting in xylol colophonium. The result is a beautiful differentiation showing nuclei blue, *hemofuscin* dark red, *hemosiderin* black (Fig. 5). Mallory's connective tissue stain (Fig. 2), also hematoxylin and Van Gieson stains, were used to bring out the connective tissue in the cirrhotic livers.

Results

The results of our experiments are presented in concise form in Table I. The data on the copper-fed animal and its litter-mate control in each instance are placed side by side for the purpose of easy comparison. The duration of the experiments was from 21 to 105 days. In the shorter periods (21 to 60 days), the duration of the feeding was determined by the death of the copper-fed animal, while in no case did a control animal die. Of the twenty-one animals on the copper diet, sixteen died of copper poisoning. Marked changes in the liver in the way of pigmentation or fibrosis are hardly to be expected to develop in such short periods of time. Yet it will be seen that Rabbits 79, 86, 90, and 92 — animals that were on the copper diet only 21 to 29 days — showed moderate pigmentation in the form of "hemofuscin," while Rabbit 79 also developed a moderate amount of iron-containing pigment. While some pigment granules are found in the hepatic cells, by far the greater quantity occur in large Kupffer cells which have apparently agglomerated to form multinuclear giant cells. The cytoplasm of these cells is loaded with yellowish brown granules that stain red with basic fuchsin.

In each of the above animals the "net" body weight is less than that of the litter-mate control. In Rabbit 90, the difference is only slight, while in Rabbit 79 there is almost 50 per cent decrease. Likewise there is a decrease in the weight of the livers in the copper-fed animals ranging from 12 to 27 per cent, except in Rabbit 86, which shows a 12 per cent increase in weight over its litter-mate control. Study of the quantities of copper ingested by these four animals shows 2.68 gm. for Rabbit 79, while the other three consumed 4.28, 4.01 and 4.56 gm. respectively. It will be seen that the intake of copper is quite proportional to the length of time that the animals

TABLE I

Complete Data on Copper-Feeding Experiment

No.	Group	Sex	Variety	Litter No.	Days on diet	Gross body wt.	Net body wt.	Liver wt. (wet)	Liver per kg. (wet)	Copper liver as percentage control	Liver wt. (dry)	Liver per kg. (dry)	Copper ingested during exp.	Copper per 100 gm. (wet) liver	Pigments		Cirrhosis	Necrosis
															Hemofuscin	Hemosiderin		
						Kg.	*Kg.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*mg.*				
*80	Control	F	Flem. Cross	11	21	1.85	1.36	65	47.8	..	16.8	12.3	..	0.01	–	–	–	
79	Copper	F	" "	11	21	1.05	0.75	31	41.3	88.7	6.9	9.2	2.68	133.00	++	++	–	
*87	Control	F	Albino	14	24	1.59	1.25	38	30.8	..	10.3	8.2	..	0.00	–	–	–	
88	Copper	F	"	14	24	1.42	1.11	42	37.8	122.5	8.5	7.7	3.96	70.80	–	–	–	++
*85	Control	F	"	13	29	1.80	1.42	61	43.0	..	15.2	10.7	..	0.00	–	–	–	
86	Copper	F	"	13	29	1.42	1.01	49	48.4	112.6	9.8	9.7	4.28	9.68	++	–	–	
*89	Control	F	"	15	29	1.88	1.47	65	44.3	..	14.3	9.7	..	0.00	–	–	–	
90	Copper	F	"	15	29	1.41	1.14	37	32.4	73.2	7.2	6.3	4.01	115.00	++	–	–	+++
*91	Control	F	"	16	29	1.43	1.11	51	45.9	..	10.8	9.8	..	0.00	–	–	–	
92	Copper	F	"	16	29	1.24	0.93	34	36.6	79.7	7.0	7.5	4.56	128.00	+++	–	–	+++
*82	Control	F	Flem. Cross	11	30	2.18	1.70	76	44.7	..	18.5	10.9	..	0.00	–	–	–	
81	Copper	F	" "	11	30	1.09	0.77	33	42.8	95.8	6.4	8.3	6.32	128.40	+++	+	+++	
*47	Control	M	Albino	6	40	1.60	1.05	62	59.0	..	16.0	15.3	..	0.06	–	–	–	
49	Copper	M	"	6	40	1.76	1.24	29	23.4	39.7	6.3	5.1	11.24	216.00	++++	–	++	
*76	Control	F	Flem. Cross	10	40	2.69	2.24	60	26.8	..	10.9	4.5	..	0.00	–	–	–	
77	Copper	F	" "	10	40	1.28	0.97	23	23.7	88.5	4.0	3.1	5.73	77.40	++	–	–	
*50	Control	M	Albino	6	44	2.57	1.87	101	54.0	..	24.4	13.0	..	0.00	–	–	–	
48	Copper	M	"	6	44	1.77	1.18	50	42.4	78.4	11.4	9.7	13.62	90.70	+++	–	–	
*71	Control	F	Amer. Blue	8	45	2.39	1.83	97	53.0	..	25.3	10.6	..	0.01	–	–	–	
72	Copper	F	" "	8	45	1.52	1.15	28	24.3	45.9	5.7	5.0	7.38	171.80	++	+	++	

*69	Control	F	Albino	7	50	2.28	1.78	63	35.4	..	15.9	8.9	..	0.18	–	–	–	
65	Copper	F	"	7	50	1.37	1.09	48	44.0	124.4	10.2	9.3	8.96	190.50	+++	–	++	
*14	Control	M	Amer. Blue	1	60	2.39	1.87	87	46.5	..	23.1	12.4	..	0.01	–	–	–	
*13	Copper	M	" "	1	60	2.09	1.62	72	44.4	95.6	20.4	12.6	13.67	53.40	–	–	–	
*19	Control	M	Chinchilla	2	60	2.22	1.80	88	48.9	..	23.1	12.8	..	0.00	–	–	–	
*18	Copper	M	"	2	60	1.93	1.48	56	37.7	77.4	14.5	9.8	13.27	81.50	++	–	–	
*21	Control	F	"	2	60	2.10	1.66	95	57.2	..	24.3	14.7	..	0.03	..	..	No	data
*20	Copper	F	"	2	60	1.71	1.28	55	42.9	75.0	14.0	10.9	13.37	126.40	+++	+	–	
*63	Control	M	Albino	7	60	2.32	1.89	75	39.7	..	17.4	9.2	..	0.46	–	–	–	
*64	Copper	M	"	7	60	1.96	1.46	64	43.8	110.0	14.5	9.9	8.93	86.50	–	–	–	
*66	Control	F	"	7	60	2.65	2.09	89	42.5	..	19.4	9.3	..	0.37	–	–	–	
*67	Copper	F	"	7	60	1.82	1.31	78	59.5	140.0	16.4	12.5	13.93	129.00	+++	–	++	
*37	Control	F	Chinchilla	4	63	2.44	1.99	72	36.2	..	18.3	9.2	..	0.07	–	–	–	
38	Copper	F	"	4	63	1.51	1.13	39	34.5	95.4	8.6	7.6	10.81	186.20	++++	–	++	
*41	Control	F	"	5	69	2.79	2.19	104	47.4	..	27.6	12.6	..	0.04	–	–	–	
46	Copper	F	"	5	69	1.49	1.15	44	38.3	80.6	9.2	8.0	14.20	198.40	++++	+	+++	
*83	Control	F	Albino	12	70	2.61	2.11	95	45.1	..	23.2	11.0	..	0.32	..	..	No	data
84	Copper	F	"	12	70	1.35	0.98	31	31.6	70.0	6.9	7.0	11.25	237.00	++++	–	++	
*74	Control	F	"	9	74	2.66	2.09	95	45.4	..	24.1	11.5	..	0.04	–	–	–	
73	Copper	F	"	9	74	1.31	0.98	39	40.4	88.4	8.0	8.2	16.11	193.10	++++	–	++	
*40	Control	M	Chinchilla	4	105	2.84	2.37	60	25.3	..	16.3	6.9	..	0.02	–	–	–	
39	Copper	M	"	4	105	2.10	1.63	37	22.7	89.8	7.1	4.4	19.60	188.00	++++	–	–	+

* These rabbits were killed. Those without an asterisk died, presumably from copper poisoning.

were on the copper diet. The amounts of copper stored in the livers are somewhat more variable, Rabbit 79 having the lowest intake, yielded 133 mg. of copper per 100 gm. of wet liver tissue. Corresponding to this is a considerable deposit of hemofuscin and hemosiderin in the liver. For some unknown reason Rabbit 86 yielded only 9.68 mg. of copper in spite of a relatively high intake. Notwithstanding this low copper content, a moderate amount of hemofuscin was found in the liver. The other two animals showed on analysis 115 and 128 mg. of copper respectively.

Rabbit 88 is the only animal in this group that failed to show pigmentation. It was fed for 24 days, ingested 3.96 gm. of copper and stored 70.8 mg. in each 100 gm. of liver tissue. The liver was large (122.5 per cent of control), mottled in appearance and microscopically showed well marked congestion and central necrosis. The histological picture is that of a well advanced chronic passive congestion.

Of the sixteen animals fed copper acetate for 30 days or more, ten showed cirrhosis and thirteen showed pigmentation. Of the five animals in the copper-fed group on the diet for over 60 days, all showed pigmentation to an extreme degree, while four animals presented a well marked cirrhosis.

The copper ingested by the rabbits fed for 30 days or more ranged from 5.73 gm. to 19.60 gm. The latter amount was ingested by the 105-day animal. The amounts of copper ingested correspond roughly to the duration on the diet. The copper content of the various livers, however, is most interesting in relation to the presence of pigmentation and cirrhosis. In a general way, it may be stated that livers containing over 100 mg. of copper per 100 gm. of wet liver substance showed cirrhosis. There are only four exceptions to this in the animals fed for more than a month (Table II) and three of these presented necrosis of the liver tissue and at the same time heavy pigmentation. It is interesting to note that the copper-fed animals which developed necrosis failed to show increase in fibrous tissue.

Only two animals, Rabbits 13 and 64, out of the sixteen fed copper acetate for a month or more, failed to show either pigmentation or cirrhosis. The copper content of the livers in these animals was 53.4 and 86.5 mg. per 100 gm. of wet liver substance respectively. It would seem, therefore, that pigmentation fails to develop until approximately 75 to 100 mg. of copper are stored in each 100 gm. of liver tissue.

In Table II, all of the copper-fed animals are grouped together in a simplified table showing only the more important factors. This gives a more concise picture of the positive findings in a form easily comparable. It shows especially well the relation of pigmentation and cirrhosis to the ingestion and storage of copper.

TABLE II

Relation of Pigmentation and Cirrhosis to Copper Content

No.	Days on diet	Ratio Cu to control livers	Copper ingested	Copper per 100 gm. (wet) liver	Hemofuscin	Hemosiderin	Cirrhosis	Necrosis
		per cent	*gm.*	*mg.*				
79	21	88.7	2.63	133.00	++	++	–	–
88	24	122.5	3.96	70.80	–	–	–	++
86	29	112.6	4.28	9.68	++	–	–	–
90	29	73.2	4.01	115.00	++	–	–	+++
92	29	79.7	4.56	128.00	+++	–	–	+++
81	30	95.8	6.32	128.40	+++	+	+++	–
49	40	39.7	11.24	216.00	++++	–	++	–
77	40	88.5	5.73	77.40	++	–	–	+++
48	44	78.4	13.62	90.70	+++	–	–	–
72	45	45.9	7.38	171.80	++	+	++	–
65	50	124.4	8.96	190.50	+++	–	++	–
13	60	95.6	13.67	53.40	–	–	–	–
18	60	77.4	13.27	81.50	++	–	–	–
20	60	75.0	13.37	126.40	+++	+	–	–
64	60	110.0	8.93	86.50	–	–	–	–
67	60	140.0	13.93	129.00	+++	–	++	–
38	63	95.4	10.81	186.20	++++	–	++	–
46	69	80.6	14.20	198.40	++++	+	+++	–
84	70	70.0	11.25	237.00	++++	–	++	–
73	74	88.4	16.11	193.10	++++	–	++	–
39	105	89.8	19.60	188.00	++++	–	–	+

The question, raised by Flinn and VonGlahn [3] as to the production of pigmentation and cirrhosis by feeding sodium acetate, was also investigated. These authors suggest that the acetate radical is responsible for the liver changes where the acetate salt of copper is used in the feeding experiments. The photomicrograph (Fig. 3), which they show to illustrate the pigment found in the liver following sodium acetate feeding, is not at all convincing, since similar quantities of pigment may be seen in the liver cells of control rabbits, especially if the animals are relatively old. We have fed three rabbits on a diet similar to that of our copper-fed animals, except

that sodium acetate was substituted for copper acetate. Littermates of the same sex were kept as controls. The animals were kept on the sodium acetate-containing diet for periods varying from 40 to 60 days. None of these animals showed either pigmentation or cirrhosis. The control animals were likewise negative as to liver changes.

Comment

Early Lesions in the Liver: The earliest change seen in the rabbit's liver in chronic copper poisoning is a moderate enlargement of the Kupffer cells, which can be seen to contain many small golden yellow granules which stain red with basic fuchsin. Potassium ferrocyanide combined with a weak solution of hydrochloric acid fails to stain the granules. This pigment has been identified by Mallory as hemofuscin, and he believes it to be the same as the iron-free pigment described by von Recklinghausen [14] in hemochromatosis. Occasionally in copper poisoning, darker granules may be seen in the Kupffer cells and in the hepatic cells. This pigment is, no doubt, hemosiderin, since it gives the Berlin blue reaction, and it corresponds to the iron-containing pigment which is so abundant in hemochromatosis in man.

Kupffer Giant Cells and Pigmentation: In livers that show somewhat more advanced changes, the Kupffer cells are seen to be greatly enlarged and many have become detached and have moved towards the portal spaces. Here groups of six to eight cells have fused to form large multinuclear giant cells (Figs. 3 and 5). These large cells apparently form in response to the presence of the hemofuscin pigment, since the two are always associated together. In some cases considerable amounts of pigment, similar to that seen in the Kupffer cells, are found in the liver cells as well. In a general way, pigment seems to accumulate in the liver cells in preference to the Kupffer cells when the feeding experiments are of long duration, and the daily copper intake therefore reduced in amount. On the other hand, larger daily feedings of copper which produce death in 30 to 60 days tend to call forth the large Kupffer cells. As the pigment accumulates rapidly in the liver, the Kupffer cells, unable to cope with the situation, mobilize and fuse to form giant cells just as the macrophages fuse about bits of suture material or foreign bodies which it seems the individual cells are unable to dissolve.

In each of our animals in which pigmentation was present, multinuclear giant cells were the most prominent part of the picture, and contained most of the pigment. In a few cases many pigment granules were also found in the liver cells. In the former experiments on copper poisoning by Hall and Butt, in which the animals were fed over periods of 33 to 35 weeks, the greater part of the pigment was present in the liver cells, although a few of the livers contained large giant cells as well. In recent experiments by Butt,[15] in which rabbits were fed copper for more than a year, the pigment is diffusely distributed in the liver cells.

No attempt will be made in the present study to inquire into the nature of the so-called "hemofuscin" which occurs in copper poisoning except to state that Mallory believes, and the writers agree with him, that hemofuscin resulting from copper poisoning contains considerable quantities of copper. A number of investigators including Mallory,[1] Abbott,[16] and the writers, believe that hemofuscin also contains iron in bound form.

Only five of our animals showed any hemosiderin and this was in small amounts. In nearly every case in which this pigment occurred, it was found in both the liver cells and the Kupffer giant cells.

Experimental Cirrhosis: In the original experiments by Mallory, *et al.*,[1] three copper-fed animals out of twenty-two developed cirrhosis. The periods of time required on the copper diet were 5½, 6½, and 11½ months respectively. Hall and Butt [2] obtained fibrosis of mild degree in a number of animals fed for periods of 8 to 9 months. The writers have obtained a cirrhosis in nine out of twenty-one copper-fed animals. If the five animals that were on the copper diet for a period less than thirty days be omitted, because of the short time, the incidence of cirrhosis is very high, amounting to 53 per cent. Furthermore, the cirrhosis is very definite, resembling human cirrhosis closely, as can be seen by referring to Figs. 2, 4 and 6. In the sections stained with hematoxylin and eosin, many giant cells loaded with yellow pigment granules may be seen scattered throughout the fibrous tissue (Figs. 4 and 6), while only occasional giant cells are seen elsewhere. This association of the pigment with the fibrous tissue, and the fact that the pigment precedes the development of the fibrosis, are presumptive proofs that the presence of the pigment stimulates the growth of connective tissue. A somewhat analogous condition may be found in the various pneumoconioses due to extraneous pigmentation of the lungs.

Conclusions

In consideration of the well controlled experiments here presented, in which seventeen out of twenty-one copper-fed animals showed pigmentation, many of them to a most extreme degree, and in view of the fact that nearly 50 per cent of this same group showed cirrhosis (this also being well marked and indisputable) we must conclude, in spite of the adverse reports by Flinn and VonGlahn,[3] Polson,[5] Herkel[6] and others, that copper poisoning in rabbits produces pigmentation and cirrhosis of the liver in a high percentage of cases if adequate doses are given. This conclusion is in accord with the original work of Mallory[1] and with the later investigation of Hall and Butt.[2]

We have found in most cases, at least under the conditions of our experiments, that 75 to 100 mg. of copper must be stored for every 100 gm. of wet liver tissue before pigmentation and cirrhosis result. The latter process apparently requires more time, and larger amounts of copper must be present in the liver tissue than are required to bring about pigmentation alone.

That large quantities of copper are stored in the liver of the copper-fed animals as compared with that found in the controls, is very evident on examination of Table I.

None of our animals received carrots, mangel-wurzels, turnips, cabbage or other plant substance rich in carotin, therefore the question of pigmentation due to these substances does not enter as a factor in the results presented here. The question of whether or not heavy feeding with carrots does produce pigmentation will be considered in a subsequent study.

The resemblance of the lesions described in this paper to those of early hemochromatosis in man is very striking.

Summary

1. The results of a study of copper poisoning in rabbits are presented in which the experiments were rigidly controlled. A littermate control of the same sex was fed a special control diet for each animal that received copper.

2. Twenty-one rabbits were fed on a diet containing 2 mg. of normal copper acetate in each gram of food. The copper salt was

added to the control diet, which consisted of 75 parts of whole ground alfalfa and 25 parts of whole ground barley.

3. The duration of the experiment was from 21 to 105 days.

4. Seventeen of the copper-fed animals showed pigmentation of the liver, mostly in the form of hemofuscin stored in Kupffer giant cells.

5. Nine of the twenty-one copper-fed animals showed cirrhosis of the liver.

6. Five of the animals, which failed to show cirrhosis, showed varying degrees of liver cell necrosis.

7. Three animals in which sodium acetate was substituted for the copper salt failed to show changes in their livers.

REFERENCES

1. Mallory, F. B., Parker, Frederic, Jr., and Nye, R. N. Experimental pigment cirrhosis due to copper and its relation to hemochromatosis. *J. Med. Res.*, 1921, **42**, 461.

2. Hall, E. M., and Butt, E. M. Experimental pigment cirrhosis due to copper poisoning. *Arch. Path.*, 1928, **6**, 1.

3. Flinn, F. B., and VonGlahn, W. C. A chemical and pathologic study of the effects of copper on the liver. *J. Exper. Med.*, 1929, **49**, 5.

4. Editorial. Controlled experimentation, hemochromatosis and the alcohol problem. *J. A. M. A.*, 1929 (Feb. 2), **92**, 393.

5. Polson, Cyril J. Chronic copper poisoning. *Brit. J. Exper. Path.*, 1929, **10**, 241.

6. Herkel, Walter. Über die Bedeutung des Kupfers (Zinks und Mangans) in der Biologie und Pathologie. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1930, **85**, 513.

7. Ellenberger and Hofmeister. (Cited from Herkel.) *Arch. f. wissensch. u. prakt. Tierh.*, 1883, **9**, 325.

8. Filehne, W. (Cited from Herkel.) *Deutsche. med. Wchnschr.*, 1895, **21**, 297; 1896, **22**, 145.

9. Baum and Seeliger. (Cited from Herkel.) *Arch. f. wissensch. u. prakt. Tierh.*, 1896, **22**, 194.

10. Oshima, F., and Siebert, P. Experimentelle chronische Kupfervergiftung. Ein Beitrag zur Frage der Pathogenese der Hämochromatose. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1930, **84**, 107

11. Schönheimer, R. Personal communication.

12. Adrianoff, N., and Ansbacher, S. Leber und Kupfer. *Deutsche. med. Wchnschr.*, 1930, **56**, 357.

13. Elvehjem, C. A., and Lindow, C. W. The determination of copper in biological materials. *J. Biol. Chem.*, 1928, **81**, 435.

14. von Recklinghausen. Ueber Hämochromatose. *Tagebl. d. Versamml. deutch. Naturf. u. Aerzte*, 1889, p. 324.

15. To be published.

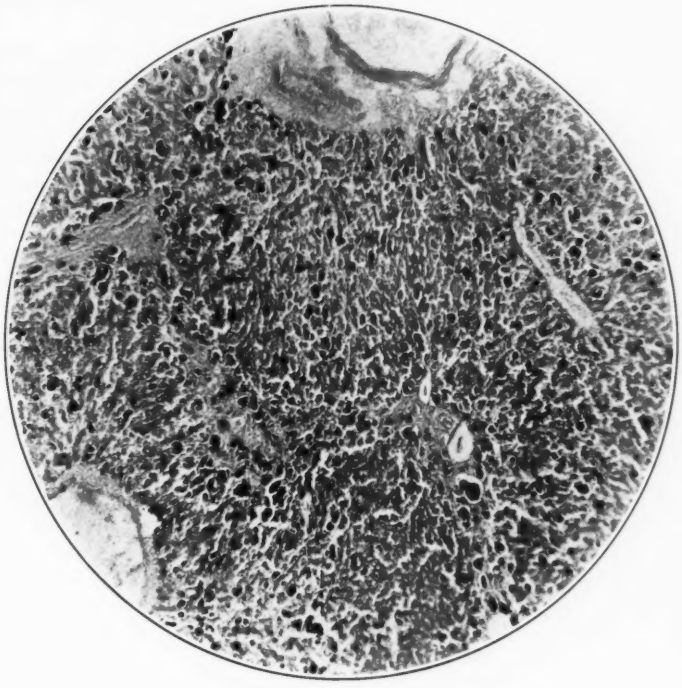
16. Abbott, Maude. Pigmentation of cirrhosis of the liver in a case of hemochromatosis. *J. Path. & Bact.*, 1901, **7**, 55.

DESCRIPTION OF PLATES

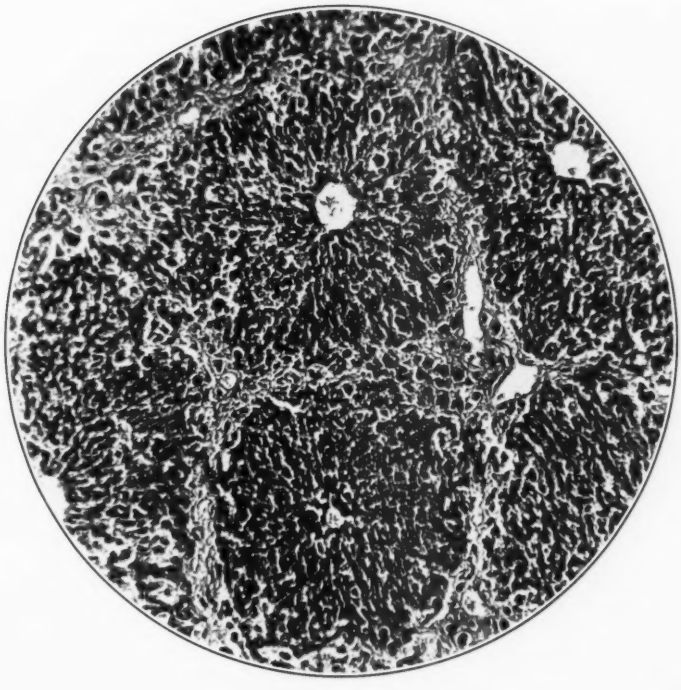
PLATE 60

FIG. 1. Rabbit 84, fed copper acetate for 70 days. Photomicrograph of liver (low power) showing numerous Kupffer giant cells filled with hemofuscin. The pigment is somewhat more abundant about the periportal spaces. Basic fuchsin stain.

FIG. 2. Rabbit 49, fed copper acetate for 40 days. Liver showing well developed cirrhosis with new fibrous tissue surrounding the lobules. Many pigment-containing giant cells in the connective tissue. Mallory's connective tissue stain.



1

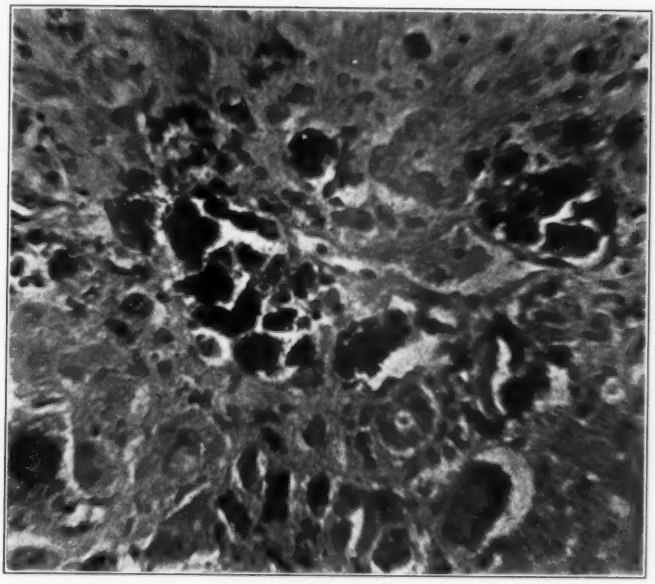


2

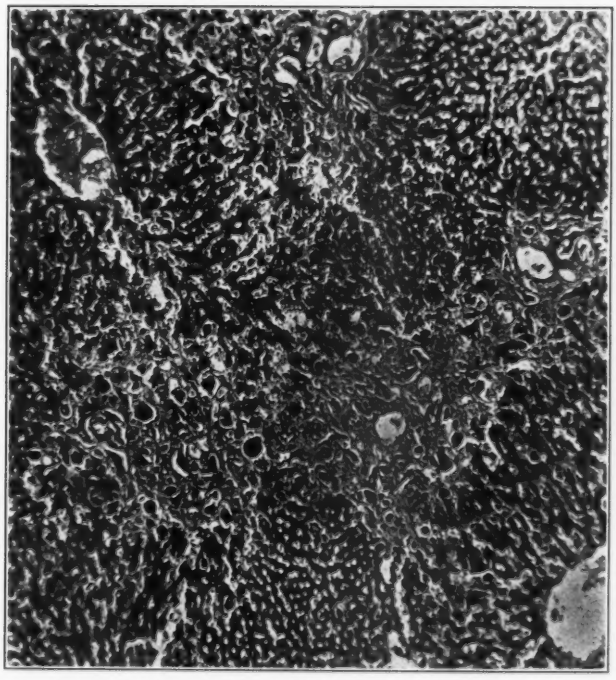
PLATE 61

FIG. 3. Rabbit 46, fed copper acetate for 69 days. High power photomicrograph of liver showing numerous Kupffer giant cells loaded with hemofuscin. Basic fuchsin stain.

FIG. 4. Rabbit 81, fed copper acetate for 30 days. Low power photomicrograph showing marked development of fibrous tissue in which many large pigment giant cells occur. Hematoxylin and eosin stain.



3

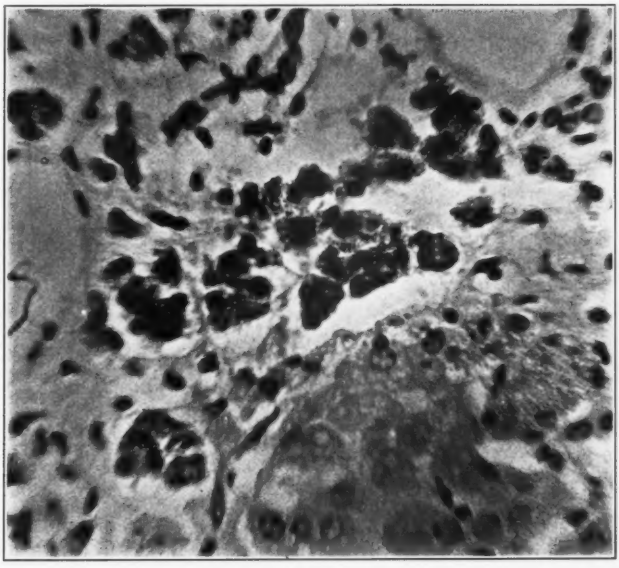


4

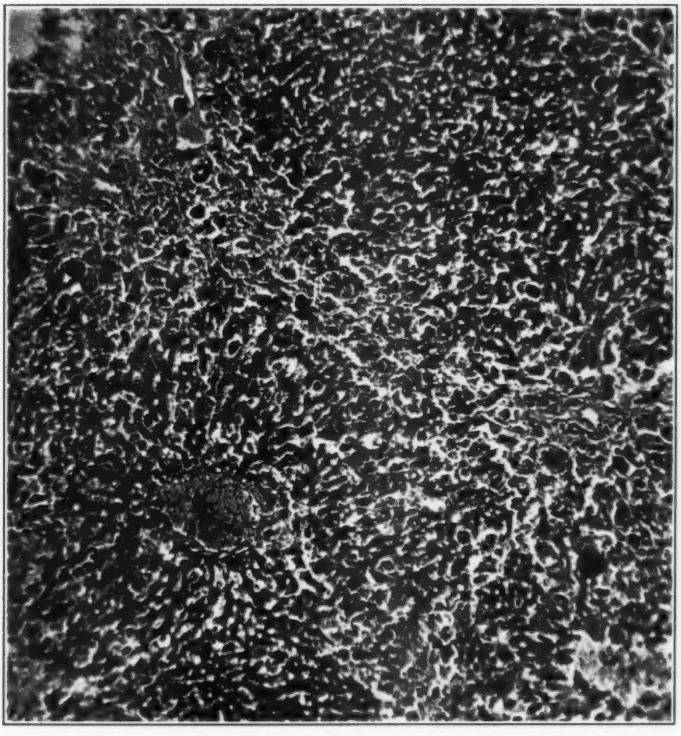
PLATE 62

FIG. 5. Rabbit 38, fed copper acetate for 63 days. Photomicrograph of liver under oil showing large Kupffer cells loaded with hemofuscin. Hematoxylin, ammonium sulphid and fuchsin stain.

FIG. 6. Rabbit 38, showing a low power photomicrograph of the liver with the development of a fine intralobular cirrhosis. Hematoxylin and eosin stain.



5



6

EXPERIMENTAL HEPATIC PIGMENTATION AND CIRRHOSIS *

II. The Effect of Heavy Carrot-Feeding on the Rabbit's Liver

Ernest M. Hall, M.D., and Eaton M. MacKay, M.D.

(From the Department of Pathology, University of Southern California, School of Medicine, Los Angeles, and The Scripps Metabolic Clinic, La Jolla, California)

Flinn and VonGlahn [1] have recently claimed that a diet consisting exclusively of carrots will produce marked pigmentation of the liver in rabbits. In fact, they state that pigment production is more readily produced by the feeding of carrots than by the feeding of copper. Although they present no data to prove the nature of this pigment, they say that it is in every way identical with that found in copper poisoning. Polson,[2] working in Great Britain, reports having obtained pigmentation of the livers of rabbits in 88 per cent of his animals by feeding carrots, oats and thirds. He describes the pigment as hemofuscin.

Buckley, Joss, Creech and Couch [3] have recently (1930) reported a condition in cattle which they designate "carotenosis of bovine livers." This condition was discovered in the course of meat inspection at five different slaughtering centers ranging from Buffalo, N. Y., to Phoenix, Arizona. The livers of these animals were found to be an intense yellow or reddish yellow color. When the fresh livers are sectioned the knife and fingers are stained a deep yellow. As the condition advances fibrous changes develop in the liver, in the more severe cases culminating in advanced cirrhosis. No pigment deposit in the form of granules or larger solid particles is described. The condition appears to be confined to the liver, as all other organs were normal in their animals. Chemical studies of the livers yielded a substance which was identified as "carotene" by chemical and boiling point determinations and spectrophotometric measurements. Carotin in amounts up to forty times that found in the normal bovine liver was obtained.

* Received for publication June 2, 1931.

Experiments were carried out in which a number of white rats were fed on the yellow beef livers. The rats developed parenchymatous degeneration and necrosis of the liver cells with more or less round cell infiltration. Since, according to Wells and Hedenburg,[4] Connor [5] and others, carotin is considered non-toxic, Buckley and his associates thought that some unknown toxic agent was the real offender, while carotin had accumulated in the liver due to damage to the liver cells.

The investigations of Buckley, *et al.*, are presented here at some length because the photomicrographs which they publish are so strikingly like the histological pictures seen in some of our rabbit livers following a diet heavy in carrots.

It has seemed desirable in view of the claims of Flinn and Von Glahn and of Polson to investigate the effect of carrot-feeding on the livers of rabbits. The necessity of adequate controls in experiments of this kind has been emphasized in the preceding paper.[6]

Methods

As before, litter-mates of the same sex have been compared, the only difference in the treatment of the two animals of each pair being a high percentage of carrots in the diet of one series. The various diets which were used are detailed in Table I.

Alfalfa bloom is prepared by grinding the dried leaves of the alfalfa plant, the stems and stalks being discarded. The fresh carrots used were the ordinary field variety which are rich in carotin. They were washed with a stiff brush until free of dirt and most of the skin. In the first experiment summer carrots were used and in the second winter carrots. There was no obvious difference between them. The dried carrot was a high-grade product prepared by drying sliced carrots in a current of recirculated air at a relatively low temperature.

The rabbits were killed in four different lots after the experiment had been in progress 32, 42, 47, and 50 days respectively. The various organs were examined grossly and blocks of liver tissue were fixed in 95 per cent alcohol. The blocks were embedded in paraffin and the sections stained with a weak solution of basic fuchsin following treatment with potassium ferrocyanide and hydrochloric acid. Sections were also stained with hematoxylin and eosin and with eosin-methylene blue. The pigment granules in the Kupffer giant cells were stained more satisfactorily with the methylene blue than with the basic fuchsin.

Four apparently normal albino rabbits were fed from the time they were 60 until they were 107 days of age, a period of 47 days, on Control Diet No. 1. Four litter-mates of the same sex received Carrot Diet No. 1 over precisely the same period. The results of this experiment are presented in Table II.

TABLE I

Composition of the Various Diets Employed

Experiment	Diet	Per cent
Control Diet No. 1	Alfalfa bloom	25
	Water	75
Carrot Diet No. 1	Alfalfa bloom	20
	Ground fresh carrot	80
Control Diet No. 2	Alfalfa bloom	75
	Ground barley	25
Carrot Diet No. 2	Fresh carrot (plus barley)	100
Dried Carrot Diet	Dried ground carrot	90
	Alfalfa bloom	5
	Ground barley	5

Nine rabbits 60 days of age were started on each of the last three diets described in Table I. For each rabbit on Control Diet No. 2 there was a litter-mate of the same sex upon Carrot Diet No. 2 and another on the Dried Carrot Diet. A third of the rabbits in each group were killed in 32 days, a third in 42 days and the remainder after 50 days on the diets. The rabbits on the fresh carrot diets received only washed or peeled fresh carrots with the addition on the 1st, 6th, 12th, 22nd and 35th days of feeding of 100 grams of oats for each three rabbits.

An interesting but as yet unexplained observation is the increase in the size of the liver of the rabbits on the fresh carrot diets. This is well shown in the averages comprising Table IV and is the most evident in those rabbits which received fresh carrots for the longest periods. In the first experiment (Table II) the mean quantity of liver per kilogram of net body weight is 58 grams while the average of those receiving chiefly fresh carrot is 96 grams.

TABLE II

Summary of Results in Carrot-Feeding Experiment No. 1, using Control Diet No. 1 and Carrot Diet No. 1

No.	Diet group	Sex	Variety	Litter No.	Days on diet	Gross body weight	Net body weight	Liver weight	Liver per kg.	Pigments		Cirrhosis
										Hemofuscin	Hemosiderin	
						Kg.	*Kg.*	*gm.*	*gm.*			
102	Control 1	F	Albino	1	47	2.12	1.58	80	50	..	..	..
107	Control 1	M	Albino	2	47	2.27	1.47	96	65	+	−	−
108	Control 1	M	Albino	3	47	1.28	0.85	51	60	±	++	−
109	Control 1	M	Albino	3	47	1.68	1.24	71	57	..	..	..
103	Carrot 1	F	Albino	1	47	1.52	1.14	70	61	−	−	−
105	Carrot 1	M	Albino	2	47	1.06	0.52	85	163	+	−	−
112	Carrot 1	M	Albino	3	47	1.80	1.30	95	79	−	−	−
113	Carrot 1	M	Albino	3	47	1.96	1.28	101	80	+	±	−

GROSS CHANGES IN THE LIVER

The only gross changes evident in the livers of the carrot-fed group were the increase in the size of the livers in the group fed on fresh carrots and the yellow to yellowish red color of the livers. There was no evidence grossly of fibrous tissue changes.

HISTOLOGICAL CHANGES IN THE LIVER

In the first experiment (Table II) practically no pigmentary changes were found. One of the controls, Rabbit 108, showed a moderate number of hemosiderin granules in the liver cells, as shown by the Berlin blue reaction. One of the carrot-fed group, Rabbit 113, showed a trace of hemosiderin. Two of the controls and two of the carrot-fed group showed a few granules of hemofuscin in the liver cells, no more than occurs physiologically in many rabbit livers. No fibrous tissue proliferation was found except that produced about the bile ducts in the coccidia-infested animals. It is evident that these results are quite different from those produced under similar conditions by the feeding of copper acetate.[6]

TABLE III

Summary of Results in Carrot-Feeding Experiment No. 2, using Control Diet No. 2, Fresh Carrot Diet No. 2, and Dried Carrot Diet

No.	Diet group	Sex	Variety	Litter no.	Days on diet	Gross body weight	Net body weight	Liver weight	Liver per kg.	Pigments		Histology	
										Hemofuscin	Hemosiderin	Cirrhosis	Other changes
						Kg.	*Kg.*	*gm.*	*gm.*				
114	Control 2	M	Albino	1	32	1.70	0.45	59	47	–	–	–	None *
123	"	M	"	2	32	1.62	0.50	55	50	–	–	–	None
126	"	M	"	3	32	1.40	0.40	52	52	–	–	–	None
117	"	F	"	1	42	1.55	0.40	57	50	–	–	–	Coarsely granular cytoplasm
120	"	F	"	2	42	1.90	0.45	65	45	–	–	–	None
127	"	F	"	3	42	1.60	0.40	55	46	–	–	–	Vacuolate cytoplasm
132	"	F	"	4	50	1.90	0.50	67	50	–	–	–	Cytoplasm granular
134	"	F	Brown	5	50	1.45	0.38	54	50	–	–	–	Coarsely granular cytoplasm
136	"	M	Black	6	50	1.55	0.40	54	47	+	–	–	Coarsely granular cytoplasm
115	Fresh Carrot 2	M	Albino	1	32	0.98	0.35	21	33	+	+	–	Granular cytoplasm, liver cells swollen
124	" "	M	"	2	32	1.15	0.37	45	57	+	–	+	Few Kupffer giant cells, fatty infiltration
128	" "	M	"	3	32	1.44	0.43	41	41	+	++	++	Large Kupffer giant cells, fatty infiltration
118	" "	F	"	1	42	0.95	0.38	36	62	–	–	–	None
121	" "	F	"	2	42	1.40	0.40	69	69	–	–	–	Vacuolate cytoplasm
129	" "	F	"	3	42	1.40	0.39	56	55	–	–	–	Vacuolate cytoplasm
133	" "	F	"	4	50	1.70	0.45	103	81	–	–	–	Vacuolate cytoplasm, early nuclear changes
137	" "	F	Brown	5	50	1.55	0.40	82	75	–	–	–	Vacuolate cytoplasm, early nuclear changes
139	" "	M	Black	6	50	1.28	0.33	52	55	–	–	–	Vacuolate cytoplasm
116	Dried Carrot	M	Albino	1	32	1.32	0.45	52	59	+	–	–	Kupffer cells slightly enlarged
125	" "	M	"	2	32	1.40	0.45	46	48	–	–	–	None
130	" "	M	"	3	32	1.77	0.55	75	62	–	–	+	None
119	" "	F	"	1	42	1.00	0.35	36	55	+	–	–	Kupffer cells slightly enlarged, occasional mitoses
122	" "	F	"	2	42	1.30	0.45	50	60	–	–	–	Coarsely granular cytoplasm
131	" "	F	"	3	42	1.40	0.40	62	62	–	+	–	Granular, vacuolate cytoplasm
135	" "	F	"	4	50	1.55	0.45	74	67	. .	. .	–	Coarsely granular cytoplasm
138	" "	F	Brown	5	50	1.42	0.36	58	54	–	–	–	Vacuolate cytoplasm, nuclei pale staining
140	" "	M	Black	6	50	1.50	0.38	62	56	–	–	–	Coarsely granular cytoplasm

* Practically all of the livers show more or less coccidiosis.

In Experiment 2 (Table III) one control and five carrot-fed rabbits show small numbers of iron-free granules in the liver or in large Kupffer cells. Rabbits 124, 128 and 116 show a number of Kupffer giant cells composed of several agglomerated cells. These cells contain a moderate number of yellowish brown granules which do not stain well with basic fuchsin but stain quite readily with methylene blue (Fig. 3). The production of Kupffer giant cells is quite similar to the early lesion produced in the rabbit's liver by the ingestion of copper salts.[6]

TABLE IV

Showing the Mean Relative Increase in Liver Weight of the Rabbits Fed on Fresh Carrots as Compared with the Controls and Those Fed on Dried Carrots

(Grams Liver per Kg. of Net Body Weight)

Days on diets	Control Diet No. 2	Fresh Carrot Diet No. 2	Dried Carrot Diet
32	49	44	56
42	47	62	59
50	49	70	59

The number and size of the Kupffer giant cells in the carrot-fed animals are greatly reduced as compared with similar giant cells produced by the copper-fed animals in the same periods of time. The giant cells in the carrot-fed group are scarcely discernible under the low powers of the microscope, while in the case of the copper-fed animals, immense cells loaded with pigment can be seen in great numbers under this magnification. An idea of the relative amounts of pigment in the livers of the carrot-fed and copper-fed rabbits may be obtained by comparing Fig. 3 with Figs. 1, 3 and 5 of our previous paper.[6] Of the twenty-two rabbits fed on the various carrot diets, only three developed giant cells containing pigment, while seventeen out of twenty-one copper-fed rabbits developed Kupffer giant cells heavily loaded with hemofuscin.

A moderate amount of hemosiderin was also seen in the liver of Rabbit 128. Blue granules were found in the hepatic cells as well as in the giant Kupffer cells. This animal likewise presented a fairly definite cirrhosis and fatty infiltration of the liver cells (Fig. 1). This is the only animal in the entire group of 22 that received carrots which shows a definite early cirrhosis. In Rabbits 124 and 130 a beginning fibrous proliferation can be discerned.

Animals fed on fresh carrots showed greater changes in the liver than did those fed on the dried substance. This may have been due to the greater consumption of the fresh vegetable.

Comment

Besides the pigmentary and fibrous changes described, many of the livers presented more or less disturbance of the cytoplasm of the hepatic cells. In the greater number of animals the cytoplasm appears to be more coarsely granular than is usually seen. Since four of the control animals show a similar condition, no great emphasis can be placed on this finding. In a number of animals, *viz.*, 118, 121, 129, 133, 137, 131 and 138, the cytoplasm is vacuolated, resembling the effects of a fatty or hydropic degeneration (Fig. 2). One of the controls, Rabbit 127, shows a similar change. It will be noted (Table III) that four of the fresh-carrot group, two of the dried-carrot and one control exhibit vacuolation of the cytoplasm. Such a condition might easily be the result of excess glycogen* stored in the liver cells. The livers of well nourished normal rabbits quite generally show a similar although less advanced condition.

None of the carrot diets (Table I) are balanced diets and are, no doubt, deficient in certain food essentials, especially protein. The cytoplasmic changes and possibly also the fibrous tissue proliferation may be dependent upon this deficiency. Since carotin is considered by most investigators to be non-toxic it seems logical to assume that lack of certain vitamin or other food essentials may well be the active factor concerned. Whether or not this be the true explanation, it is evident that this type of feeding experiment is difficult if not impossible of interpretation. Obviously if the diet is deficient in any essential food substance no one can say whether results produced are due to the action of carotin or due to the lack of some food essential. Further investigation of the nature of the above changes is now under way.

* Best's carmine stains of the liver in one of the animals fed dried carrots shows all of the liver cells loaded with glycogen. The glycogen granules are large, vary considerably in size and quite evidently occupy the spaces in the cytoplasm which are seen in ordinary sections. The hematoxylin and eosin stain shows clear vacuolated cells similar to those shown in Fig. 2. The control liver shows considerable glycogen in the central two-thirds of the lobule with the cells of the periphery free. The granules are uniform in size and evenly distributed. One of the fresh carrot livers reveals glycogen in amounts between the control and the one fed dried carrots. Ordinary stains of the fresh carrot and control animals fail to show the vacuolate appearance of the cytoplasm.

SUMMARY

1. Twenty-two rabbits, divided into three groups, were fed on three different diets, all of them containing a high percentage of carrots.

2. Litter-mate controls were used throughout.

3. The duration of the experiments was from 32 to 50 days.

4. Three animals developed Kupffer giant cells which contained a moderate amount of hemofuscin.

5. One rabbit developed a definite early cirrhosis; two other animals showed slight connective tissue proliferation in their livers.

6. Many of the animals showed clear, vacuolated cytoplasm in the liver cells probably due to excess glycogen storage.

7. Other changes include parenchymatous degeneration, fatty infiltration and early nuclear degenerative changes. These conditions may be dependent upon diet-deficiency due to the almost exclusive diet of carrots.

REFERENCES

1. Flinn, F. B., and VonGlahn, W. C. A chemical and pathologic study of the effects of copper on the liver. *J. Exper. Med.*, 1929, **49**, 5.
2. Polson, Cyril J. Chronic copper poisoning. *Brit. J. Exper. Path.*, 1929, **10**, 241.
3. Buckley, J. S., Joss, E. C., Creech, G. T., and Couch, J. F. Carotenosis of bovine livers associated with parenchymatous degeneration. *J. Agric. Res.*, 1930, **40**, 991.
4. Wells, H. G., and Hedenburg, O. F. The toxicity of carotin. *J. Biol. Chem.*, 1926, **27**, 213.
5. Connor, C. L. Studies on lipochromes. III. The quantitative estimation of carotin in blood and tissues. *J. Biol. Chem.*, 1928, **77**, 619.
6. Hall, E.M., and MacKay, E. M. Experimental hepatic pigmentation and cirrhosis. I. Does copper poisoning produce pigmentation and cirrhosis of the liver? *Am. J. Path.*, 1931, **7**, 327.

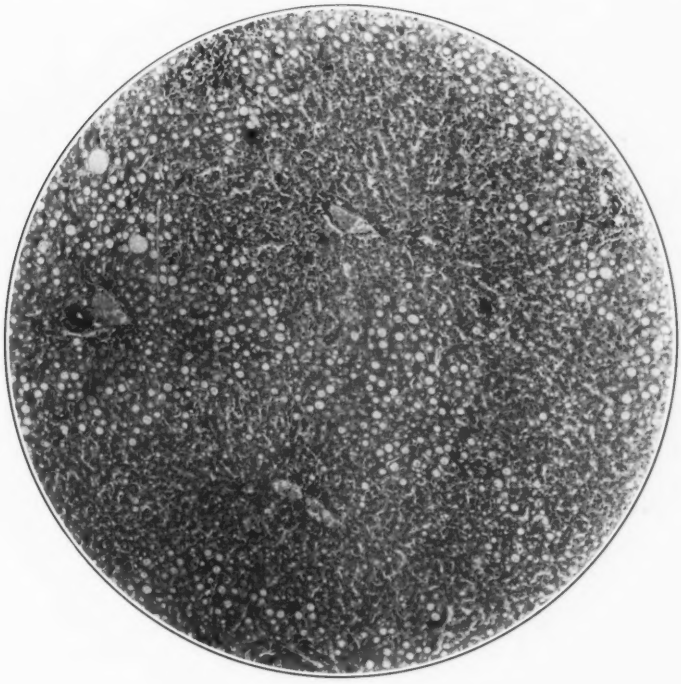
DESCRIPTION OF PLATE

PLATE 63

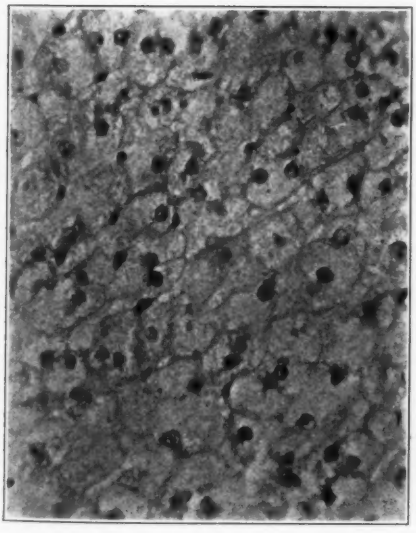
FIG. 1 Rabbit 128. Photomicrograph of liver showing early cirrhosis with fatty infiltration. The Kupffer giant cells are scarcely discernible. Hematoxylin and eosin. Low power.

FIG. 2. Rabbit 133. Photomicrograph showing swollen liver cells with clear cytoplasm and beginning nuclear changes. Hematoxylin and eosin. High power.

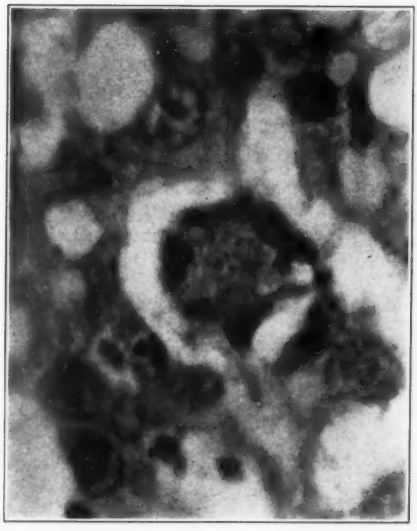
FIG. 3. Rabbit 128. Photomicrograph showing Kupffer giant cells containing 8 to 10 nuclei and a few granules of hemofuscin in the cytoplasm. Eosin and methylene blue. Oil immersion.



1



2



3

Hall and MacKay

Experimental Hepatic Pigmentation and Cirrhosis II

EXPERIMENTAL COPPER POISONING *

FRANK B. MALLORY, M.D., AND FREDERIC PARKER, JR., M.D.
(From the Pathological Laboratory of the Boston City Hospital, Boston, Mass.)

INTRODUCTION

In 1921 Mallory, Parker and Nye [1] announced that it was possible to produce pigmentation and cirrhosis of the liver in rabbits and sheep by administering to them copper salts or metallic copper in powdered form. Their results have been confirmed by Hall and Butt [2] but denied by Flinn and VonGlahn [3] and by Polson,[4] who claim that copper does not produce either pigmentation or cirrhosis and that the pigmentation present is due to a diet of carrots or similar vegetable.

Oshima and Siebert [5] and more recently Herkel [6] have attempted in vain to duplicate our results and Herkel was compelled to reach the conclusion that it is not possible to produce cirrhosis of the liver in rabbits and rats by the use of copper. The pigmentation in the rabbit's liver, he adds, occurs physiologically. At the same time he acknowledges that this natural pigment is not identical with that in the livers of our rabbits, sections of which he has studied, and states that we have without doubt produced in certain animals severe changes in the liver in the sense of degeneration followed by connective tissue proliferation.

What is the cause of these two diametrically opposed views; one, that copper produces pigmentation and cirrhosis of the liver in rabbits; the other, that it produces neither? It may depend on one of two different factors: first, that the dose of copper which actually gets into the tissues is too small, the other, that there are breeds of rabbits in which, as in guinea pigs, the blood and liver are apparently immune to the action of copper.

Proof that the natural pigment occurring in the livers of rabbits and that due to the toxic action of copper are entirely different chemically would necessarily settle immediately one of the points in dispute. One of the objects of this paper is to present such proof.

* Presented April 3, 1931, before the American Association of Pathologists and Bacteriologists at Cleveland, Ohio.
Received for publication May 27, 1931.

Experimental Work

For the past two years we have been studying the lesions produced by acute and chronic poisoning with copper, paying attention especially to the origin and nature of the pigmentation which always occurs in the liver. The animals used were rabbits, sheep, monkeys and guinea pigs.

The copper was administered in the form of metallic powder (electrolytic, Eimer & Amend) suspended in lard warmed before use to body temperature, and was injected subcutaneously by means of a syringe and needle. The lard and body juices readily dissolve the powder at a sufficiently rapid rate for the needs of the experiment. The method is much simpler, surer and more accurate than putting powder on food or using any of the copper salts and introducing them through a stomach tube or mixed with the food. As the suspension ages it acquires a bluish green color due to solution of copper and is on this account more toxic than a suspension freshly made. This point should be borne in mind.

A 20 per cent suspension of the metallic powder in lard was ordinarily used. 1 cc. therefore equalled 200 mg. of copper. The dose varied from this up to 1 gm. and was repeated every four weeks if the animal survived. It was found advisable to inject the dose in small amounts at several different sites so as to avoid the formation of a sterile abscess and sloughing. Young rabbits were used and averaged about 1500 gm. in weight at the beginning of the injections. A dose of 1 gm. usually killed a rabbit with acute lesions in two to four weeks, but one rabbit which received one dose of 2 gm. survived eight weeks.

It cannot be too strongly emphasized that a sufficient dose of copper must be administered in order to reproduce our results. If a certain minimum is not exceeded, the animal can handle it with no injurious effects. As examples of the importance of dosage, we cite the records of two animals in our series. The first is a rabbit which had been receiving copper in the form of a solution of the acetate on its food daily for three years and two months. At that time a piece of liver was removed for microscopic study and was found to show a considerable number of coarse pigment granules at the peripheries of the lobules, but no cirrhosis or necrosis. The animal was then given powdered metallic copper sprinkled in generous

amounts on its food each day. Nine months later it died and showed extensive pigmentation and cirrhosis and tubular nephritis. The second animal, a monkey, was injected at monthly intervals with 2 cc. of a 4 per cent suspension of copper over a period of seven months. A piece of liver was then removed and was completely negative microscopically. The dose administered was increased to 5 cc. of a 20 per cent suspension of copper. The animal died nine months later and its liver then showed extensive pigmentation, the pigment occurring in liver cells and macrophages.

Sheep are so susceptible to copper that a 4 per cent suspension had to be used for them in order to make the dose small enough (1 cc. equals 40 mg.).

Acute Poisoning

If copper is given in a sufficiently large dose, it will kill a rabbit in from twenty-four hours to two or three weeks. We have considered any animal dying within three weeks after injection as dying of acute poisoning. In our description of the pathological changes, we shall consider the blood, bone marrow, spleen, kidney and liver in the order named, reserving a separate section for discussion of pigmentation.

Blood, Bone Marrow and Spleen: In rabbits, twenty-four to forty-eight hours after the injection of copper, there occurs a fall in the red blood cell count accompanied by a corresponding fall in the hemoglobin and a rise in the reticulocytes. The lowest level of the erythrocyte count is usually reached four days after the injection, the number of red cells varying from two and a half to three million per cmm. with a similar reduction in the hemoglobin. At this time, the reticulocytes show a marked rise, often reaching 20 or 30 per cent, in one instance 41 per cent, and it is not unusual to find an occasional nucleated red blood cell. Following this period, the erythrocyte count and hemoglobin tend to return gradually to a normal level, accompanied by a decrease in the number of reticulocytes. This normal level is reached in two to four weeks. Smears of the blood show no stippling or other abnormalities of the red cells except for the rather large basophilic cells, the reticulocytes.

Spectroscopic examination of the serum or plasma at the height of the anemia has consistently failed to reveal any significant hemoglobinemia; the trace of hemoglobin present is probably to be accounted for by trauma incident to taking the sample of blood.

The leucocyte count tends to be low, averaging 3000 to 6000 cells per cmm. There is often a decrease in the number of polymorphonuclear cells accompanied by a rise in the monocytes. An occasional metamyelocyte can sometimes be found.

In contrast to rabbits, guinea pigs develop no anemia, even if the dose of copper be such that the animal dies in a few days. There seems to be a tendency to a rise in the reticulocytes and, in some instances, an increase instead of a decrease in the hemoglobin and erythrocytes.

The blood of two monkeys was examined three days after the injection of copper. One animal showed a drop of 600,000 red cells and the other a decrease of 1,800,000 red cells per cmm. below the counts taken before injection. In both animals, the reticulocytes were more than doubled.

The bone marrow of rabbits dying at various times after injection with the metal never shows any degeneration or necrosis. In the acute stages, there is usually marked activity of both the erythroblastic, granulocytic and megakareocytic series.

The spleens on the whole from our series of animals have shown no definite pathological changes. However, the spleen from a rabbit killed forty-eight hours after injection and with an anemia of 2,080,000 red cells per cmm. showed many clumps of non-hemoglobin-containing red cells. Most of these clumps occurred free in the pulp but a certain number had been phagocyted by macrophages.

Kidney: A certain percentage of our animals had hemoglobinuria at the time of death. The urine from such animals was dark brown in color. Spectroscopic examination revealed a large amount of oxyhemoglobin and a trace of methemoglobin. Histologically the kidneys showed numerous casts in the tubules. These casts varied in color from greenish to red in sections stained with eosin-methylene blue. In structure they were solid, hyaline casts or were hollow cylinders or a series of globules approximately the size of red blood corpuscles. We do not know the composition of the material composing these casts although in the past it has been assumed to be hemoglobin. This material was not found in the capsular spaces of the glomeruli. Copper could not be demonstrated in such casts. Associated with the occurrence of these casts, and the concomitant hemoglobinuria, was necrosis of the tubular epithelium.

In a certain number of animals that showed no hemoglobinuria at the time of death, similar casts could be found in groups of tubules in the cortex, undoubtedly representing a late or healed stage of hemoglobinuria. Experiments on phenylhydrazine poisoning support this explanation.

In kidneys of rabbits showing neither hemoglobinuria nor necrosis of the tubular epithelium, evidence of injury to the epithelium of the convoluted tubules was present in the form of hyaline or colloid droplets in the cytoplasm of the cells.

In the guinea pigs in our series, hemoglobinuria occurred in only one animal and that was one that died forty-eight hours after injection. Necrosis of the tubular epithelium in guinea pigs dying of acute poisoning was not uncommon.

In the other species of animals employed in our experiments, that is, monkeys, sheep and rabbits, hemoglobinuria was quite common and especially so in the sheep.

Liver: The liver in acute poisoning shows two lesions of importance — pigmentation and necrosis. As early as twenty-four hours after injection, a considerable number of pigment granules can be found in the liver cells, especially at the peripheries of the lobules. These granules contain copper and form the characteristic pigment of copper poisoning, as will be discussed later. From twenty-four hours on, these pigment granules continue to increase in number until at two weeks the pigmentation is marked and can be recognized grossly as well as microscopically. Grossly such a liver is a golden brown. Microscopically the pigment granules are found in the liver cells, and, as a rule, in macrophages throughout the lobules and in the portal areas. The majority of the pigment-containing liver cells appear normal, but a certain number are degenerating and others are definitely necrotic. Many of the necrotic cells do not contain pigment, indicating that copper, and not the pigment itself, is the cause of the necrosis. The pigment in the macrophages represents that taken up during the removal of dead, pigmented cells.

Necrosis of the liver cells appears later than the pigmentation. The time of its first appearance has not been definitely determined, but two weeks after injection it is present and conspicuous. Its degree and location vary somewhat in different animals. When the injury is severe, all the liver cells at the centers of the lobules are involved, giving rise to the picture of an extensive central necrosis.

When the process is less marked, single liver cells or small groups of them scattered throughout the lobules are affected. The necrotic cells are invaded and removed by macrophages. The lesion is apparently confined to the liver cells, not affecting the bile duct epithelium or the tissues composing the stroma.

Pigmentation: The livers of normal rabbits frequently contain varying amounts of pigment in the form of yellow granules. An apparently similar pigment occurs also in large amounts in the mesenteric lymph nodes. This natural liver pigment is increased with age and also as a result of acute and chronic infections. It stains with basic dyes such as fuchsin but not with fat stains such as Sudan IV or oil red O, differing in this respect from the so-called waste pigment found in humans.

Up to the present time there has been no method of distinguishing between this natural pigment and the pigment occurring after copper poisoning and this fact has undoubtedly led to confusion and scepticism on the part of other workers who have tried to repeat our work. However, recently we have found that the application of a method which has long been known clears up this difficulty completely. The method depends on the fact that a freshly prepared solution of hematoxylin stains copper a deep blue to blue-black. The details of this are given in the section on technic.

Application of this stain revealed the fact that the pigment granules which appear in the liver cells twenty-four hours after the injection of copper contain copper. At two weeks there are numerous pigment granules which stain positively for copper. From this time on, up to two months after injection, copper could be demonstrated in the pigment granules. However, at this point the staining of the granules had become less intense, indicating that the metal was disappearing. At five months, the copper had completely disappeared from the granules and the pigment either did not stain at all or stained a brownish black, indicating the presence of iron. This was confirmed by the ferrocyanide reaction for iron. The natural pigment of the rabbit liver is not stained at all by hematoxylin.

Therefore, by means of this method, we have been able to show that copper is present in the pigment granules following the injection of copper, and that such pigment granules, after the copper leaves them, begin to show unmasked iron. These two findings would seem to answer the contention of some other workers that the pigment

observed by us was natural pigment or pigment due to certain substances in the diet.

Moreover, a corroborative microchemical method has been employed by us, namely, the triple nitrite test for copper, the technic of which is given below. This test was applied to sections of the same livers on which we used the hematoxylin stain. By means of this microchemical reaction, we have been able to demonstrate the presence of copper in sections of livers from acutely poisoned rabbits, on which the hematoxylin stain was likewise positive for copper. Thus, by means of the triple nitrite test, we showed that copper was present in such livers and, by staining with hematoxylin, that it was located in the pigment granules, and, to a less extent, in the cytoplasm of the hepatic cells. The triple nitrite test is negative with normal livers, not being sufficiently sensitive to detect the amount of copper normally present.

Chronic Poisoning

The pathology of this form of poisoning has been described in detail in our former report. In brief, the two outstanding lesions are pigmentation and cirrhosis. Our latest findings in regard to the method of production of the pigment and its characteristics have been described in the section on pigmentation. The cirrhosis is the result of repeated necrosis of the liver cells following each injection of copper and of an increase in stroma. This increase in stroma occurs partly as a result of condensation where necrotic liver cells have not been replaced and partly due to new formation of connective tissue and blood vessels to serve as stroma for islands of newly formed liver cells. Grossly, such a liver is firm and its surface is finely granular. Some of our animals which had cirrhosis showed jaundice, and in one instance ascites.

Technic

Hematoxylin Stain for Copper

1. Fix tissue, cut in thin slices, in 95 per cent alcohol or in 10 per cent formalin, buffered to a pH of 7.0.

2. Stain frozen, celloidin or paraffin sections in a freshly prepared, neutral aqueous solution of hematoxylin for one hour or longer. A solution which is acid or more than very faintly alkaline

will not stain copper well, apparently because the metal is dissolved out of its compounds.

3. Wash sections in tap water, dehydrate in alcohol, clear in origanum oil or xylol and mount in xylol balsam.

Copper compounds are stained blue to blue-black, hemosiderin brownish black to black. The stain seems to be permanent.

Note. The staining solution is made by adding a little hematoxylin (as much of the crystals or powder as will cover the point of a small scalpel) to 10 cc. of water, which must be neutral, and dissolving with the aid of heat. A strength of about 0.5 per cent is desirable. Distilled water is almost always acid and gives with hematoxylin a yellow solution.

The best and most reliable hematoxylin solution is made by using a mixture of solutions of monopotassium and disodium phosphates with a pH of 7.0. The color of the hematoxylin disolved in this buffered solution is a rich red owing to the presence of the buffer salts.

A counterstain in a 0.25 per cent solution of basic fuchsin in 50 per cent alcohol is sometimes advantageous as it brings out the granules which contain no copper or stainable iron. Stain the sections in the fuchsin solution for 5 to 20 minutes, differentiate well in alcohol, and clear and mount as before.

Triple Nitrite Test for Copper

1. Blot a frozen, celloidin or paraffin section of alcohol-fixed liver tissue on a slide.
2. Place on it a crystal of sodium acetate.
3. Add one or two drops of a saturated solution of potassium nitrite.
4. Add one or two drops of a 1 per cent solution of acetic acid.
5. Dissolve a minute amount of lead acetate in the above mixture.
6. Cover the section with a cover glass and watch under the microscope for the development of square dark yellow crystals on the surface of the tissue. As they enlarge they appear black.

Discussion

We feel that the results of acute poisoning with copper described above both throw light on the mechanism of copper poisoning and also support our former claims as to the effect of copper in producing

pigmentation and cirrhosis of the liver. We will take up first a discussion of the mode of action of copper in experimental poisoning and then review our present results in relation to our former findings.

One of the most important effects of acute copper poisoning from our point of view is the changes produced in the blood. Since we felt that the pigment formed as a result of poisoning with copper represented some form or derivation of hemoglobin, it was essential that we demonstrate that copper has an effect on the hemoglobin in the animal body. That copper hemolyzes red cells *in vitro* has long been known, but in our previous work on chronic copper poisoning, none of the animals showed anemia or other evidence of blood destruction, with the exception of hemoglobinuria. This hemoglobinuria without a preceding anemia was difficult to explain. In pigment cirrhosis in humans no anemia is present, and this fact has been utilized by some as an argument against the idea that copper has any effect on hemoglobin, such as hemolysis or destruction of red blood cells. However, the explanation of this phenomenon is doubtless the same as that which applies to our chronically poisoned animals; namely, that the action of copper in small amounts over a long period of time destroys such an insignificant number of erythrocytes each day that it is impossible to detect such a loss. It was not until we carried out our acute poisoning experiments that the action of copper on the blood was revealed.

In such acute poisoning an anemia developing within one to two days after injection is the rule. Coincident with the development of this anemia there appears in the liver a copper-containing compound in the form of pigment granules which morphologically and tinctorially resemble hemofuscin. That such pigmentation is related to the anemia can be shown by comparing the effect of acute poisoning in the guinea pig. In this animal no anemia develops and similarly no deposition of pigment occurs, a fact which would seem to demonstrate beyond question the relation of the action of copper on the blood to the pigmentation of the liver. The occurrence of copper in the pigment granules is a further point of great importance for our contention that such pigment is the result of copper poisoning and is not normal pigment or pigment derived from certain foodstuffs.

The mode of action of copper in acute poisoning would seem to be as follows: the first effect is hemolysis of a certain number of red cells leading to a liberation of hemoglobin. The evidence in favor of this

is the occurrence of hemoglobinuria, the anemia and the fact that the spleen of a rabbit forty-eight hours after injection contained masses of hemolyzed red cells. The hemoglobin thus liberated is in part excreted by the kidneys and in part taken up by the liver. That we have not been able to demonstrate a definite hemoglobinemia probably can be explained by the supposition that the hemoglobin, as fast as it is set free, is disposed of in these two ways. Since no hemoglobin can be found in the glomerular capsular spaces of kidneys containing hemoglobin casts, it must be excreted in a dilute form and concentrated in the tubules.

The copper, as fast as it penetrates the body fluids, is stored in the liver, and we have found it there twenty-four hours after injection of the metal. Herkel has shown that by far the greater part of the copper in experimental poisoning is stored in this organ. Since no copper can be demonstrated in the hemoglobin casts, it is probably absorbed as such and not as a combination with hemoglobin. Thus the metal and some form of hemoglobin or a derivative are taken up separately by the liver. In the liver cells a compound of the two substances forms, giving rise to the pigment granules characteristic of this form of poisoning.

In addition to taking part in the formation of this pigment, copper also has a toxic action on the liver cells, leading to degeneration and necrosis. It has a similar effect on the tubular epithelium in the kidney. In this organ the injurious action is not dependent on the often associated hemoglobinuria, for tubular nephritis is common in the guinea pig, an animal in which hemoglobinuria is very rare, and also tubular degeneration is an almost constant finding in the kidneys of rabbits in which no hemoglobinuria or only a healed stage of the process is present.

Copper, like lead, acts on the circulating red cells and not on the marrow. Histological examination of the marrow has shown no evidence of degeneration or necrosis, but, on the other hand, an active hyperplasia.

In the liver the copper persists for several weeks following administration, but at the end of two months it is definitely decreased in amount, a certain percentage of granules in such a liver containing no copper while others contain a much lessened amount. The copper presumably is excreted through the bile, a point that will be considered in our second paper. At the end of five months copper is no

longer demonstrable, but unmasked iron has begun to appear. The presence of this unmasked iron in the pigment granules is in favor of the supposition that the pigment granules are originally composed in part of some derivative of hemoglobin rather than of one of the bile pigments or porphyrins, as has been suggested.

In our former work we claimed that chronic poisoning with copper led to pigmentation of the liver and to cirrhosis. The pigment so formed gave all the staining reactions of hemofuscin, and in animals that had survived a sufficient period of time iron could be demonstrated, the pigment then having the characteristics of hemosiderin. Our results were denied by some and confirmed by others, and it was apparent to us that several points had to be investigated in order to establish firmly our claims.

One of the difficulties was the question of the relationship of the pigment in normal rabbit livers to that which we believed to be caused by copper poisoning. At that time, we had no means of distinguishing the two and their apparent identity quite naturally led to scepticism on the part of other workers. However, the result of the application of the method described above has settled this point beyond dispute; namely, the demonstration that the pigment following copper poisoning contains copper while the normal pigment does not. In this way a clear-cut differentiation of the two pigments has been made possible, and, equally important, it has shown the actual presence of the metal in the pigment so formed.

Another point needing elucidation to confirm our conception of the action of copper was the question of the effect of this metal on the blood. This has been discussed above. A final question was whether copper could injure the liver cells and produce cirrhosis. We have found that copper does cause necrosis of liver cells and if this effect is repeated over a sufficiently long period of time cirrhosis results.

In brief, our conception of the action of copper in experimental poisoning is as follows: copper causes hemolysis and a part of the hemoglobin so liberated is taken up by the liver. At the same time copper is absorbed by the liver and there is deposited in the liver cells a compound made up of the two substances in the form of a pigment, staining like hemofuscin and containing demonstrable copper. As time goes on, this pigment increases in amount, but after a period of several weeks following injection of the metal the copper gradually disappears from the pigment granules, presumably being ex-

creted in the bile. At this time the pigment granules stain like hemofuscin, but contain no copper or demonstrable iron. However, at a later period, unmasked iron begins to appear and the pigment takes on the characteristics of hemosiderin. In addition to causing the deposition of pigment, the copper leads to degeneration and necrosis of the liver cells, and, if continued over a long enough time, a form of pigment cirrhosis results.

It should be noted that there is a variation in the reaction of different species of animals to copper poisoning. The monkey (*macacus rhesus*) requires a larger dose of copper than the rabbit, but the lesions produced are the same. The sheep is very susceptible and death from hemoglobinuria is likely to occur if the dose is too large. Pigmentation in the liver is not so prominent, but necrosis, cirrhosis and bile stasis are conspicuous. The guinea pig has been found very resistant to the action of copper except as regards the kidney, where a tubular nephritis commonly occurs. It has not been possible to produce an anemia, and pigmentation occurs only after a long continued series of injections of copper.

Summary and Conclusions

1. Acute poisoning with copper causes anemia, hemoglobinuria, necrosis of hepatic and renal cells and pigmentation.

2. The pigment so formed is a combination of copper and some derivative of hemoglobin and can be stained with a neutral solution of hematoxylin.

3. The differential staining property of this pigment depends on its copper content.

4. The pigment granules which often occur in the rabbit's liver under natural conditions and which certain other investigators have mistaken for those due to the action of copper are not colored by this method.

5. As a result of repeated injections of copper over a long period of time, a form of pigment cirrhosis results.

REFERENCES

1. Mallory, F. B., Parker, F., Jr., and Nye, R. N. Experimental pigment cirrhosis due to copper and its relation to hemochromatosis. *J. Med. Res.*, 1921, **42**, 461.

2. Hall, E. M., and Butt, E. M. Experimental pigment cirrhosis due to copper poisoning. Its relation to hemochromatosis. *Arch. Path.*, 1928, **6**, 1.

3. Flinn, F. B., and VonGlahn, W. C. A chemical and pathologic study of the effects of copper on the liver. *J. Exper. Med.*, 1929, **49**, 5.

4. Polson, C. J. Chronic copper poisoning. *Brit. J. Exper. Path.*, 1929, **10**, 241.

5. Oshima, F., and Siebert, P. Experimentelle chronische Kupfervergiftung. Ein Beitrag zur Frage der Pathogenese der Hämochromatose. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1930, **84**, 106.

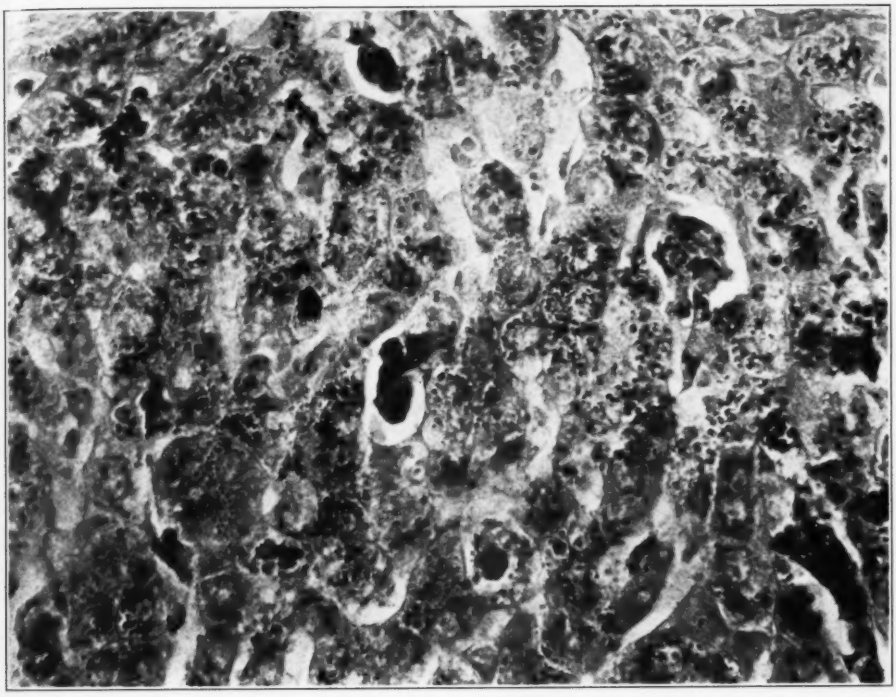
6. Herkel, W. Über die Bedeutung des Kupfers (Zinks und Mangans) in der Biologie und Pathologie. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1930, **85**, 513.

DESCRIPTION OF PLATES

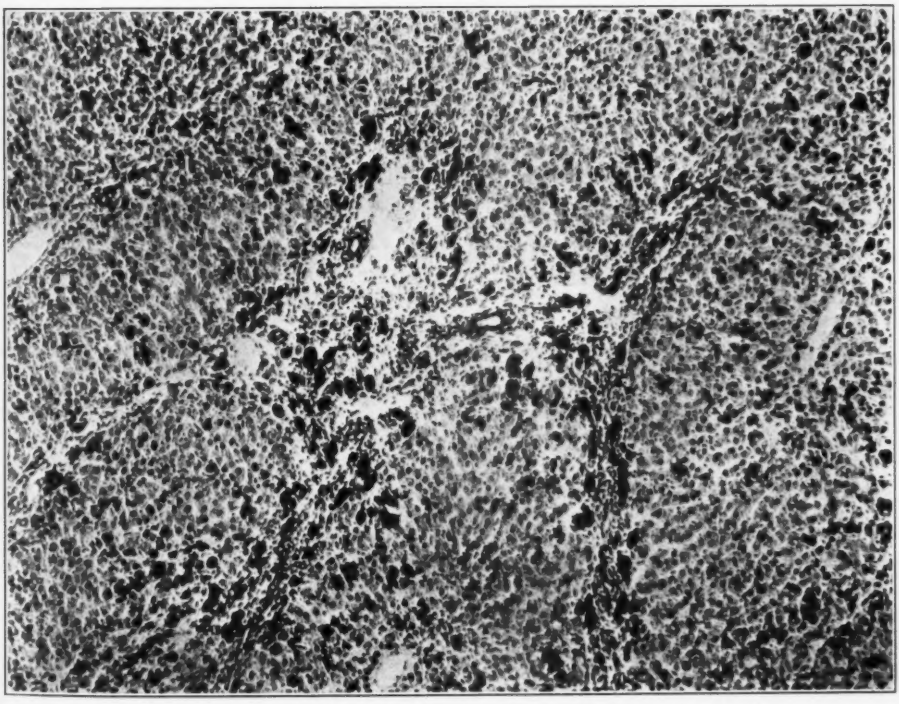
PLATE 64

FIG. 1. Liver of rabbit (Z 690) injected subcutaneously with 2 cc. of a 20 per cent suspension of powdered metallic copper in lard and killed on the fourteenth day. Numerous pigment granules present in liver cells and to some extent in macrophages. Granules colored deep blue after section was stained for one hour with neutral aqueous solution of hematoxylin. × 500.

FIG. 2. Liver of rabbit (Z 649) injected subcutaneously with 6 cc. of a 20 per cent suspension of powdered metallic copper in lard. Animal dead at end of eight weeks. Section shows numerous pigment granules in liver cells and especially in macrophages, stained deep blue by the phloxine-methylene blue method. Beginning cirrhosis evident. × 100.



1



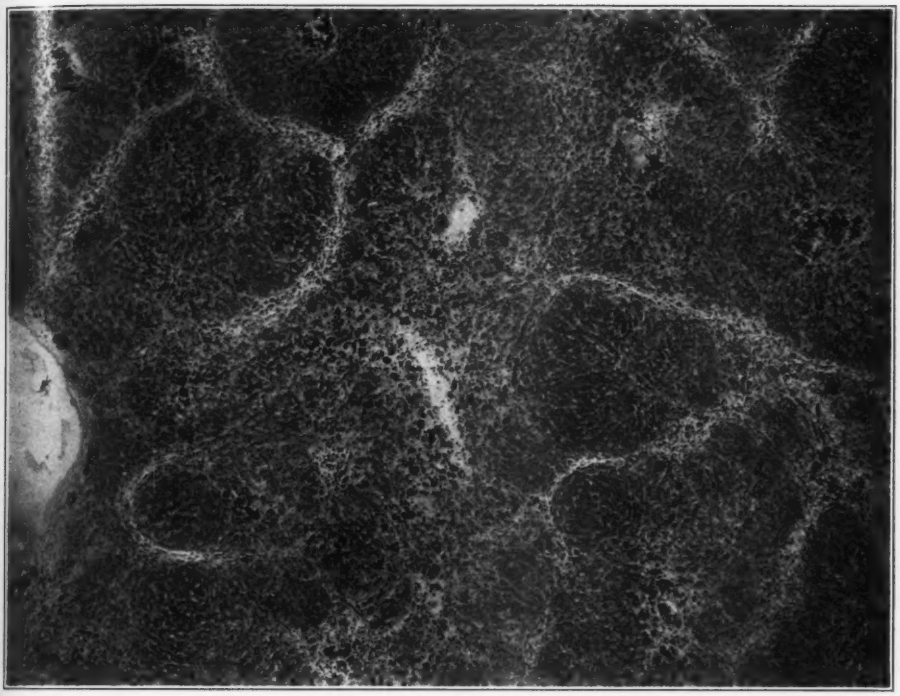
2

Mallory and Parker Experimental Copper Poisoning

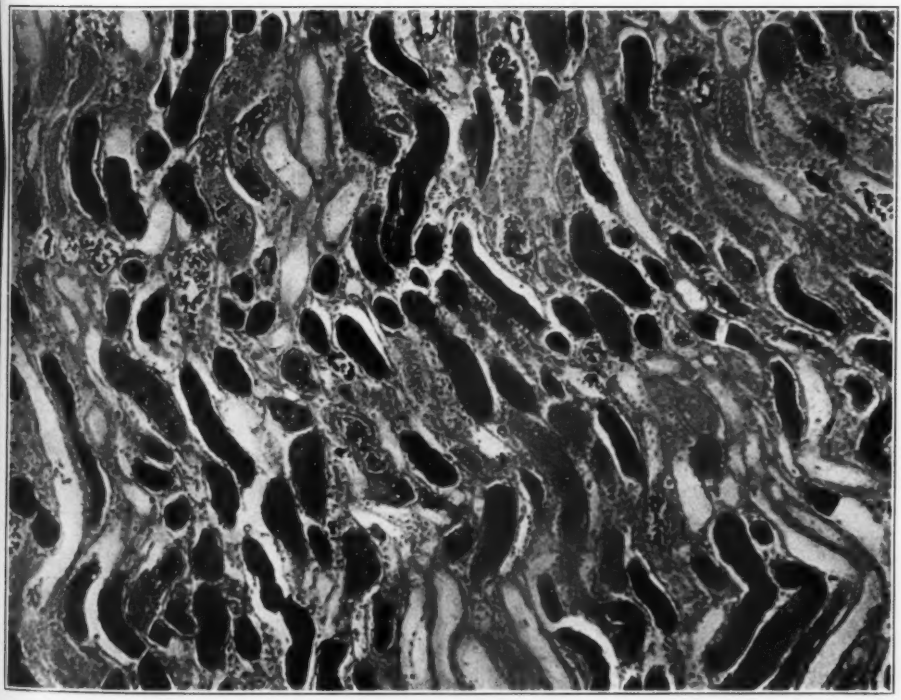
PLATE 65

FIG. 3. Liver of rabbit (Z 211) given copper acetate in moderate doses on food for three years and two months. Biopsy showed fairly numerous rather coarse pigment granules in liver cells at peripheries of lobules; otherwise negative. Metallic copper powder sprinkled on food for following nine months, when animal died. Liver large, very dark (greenish chocolate color); surface finely granular; consistence dense. Section shows pigmentation and marked cirrhosis. Phloxine-methylene blue stain. × 40.

FIG. 4. Kidney of sheep (Z 282) fed copper acetate in moderate doses on food for eleven and a third months. Killed because off feed for over a week. Kidneys large and black. Section shows great numbers of hemoglobin casts in collecting tubules. Phloxine-methylene blue stain. × 100.



3



4

Mallory and Parker Experimental Copper Poisoning

THE MICROCHEMICAL DEMONSTRATION OF COPPER IN PIGMENT CIRRHOSIS *

FRANK B. MALLORY, M.D., AND FREDERIC PARKER, JR., M.D.
(From the Pathological Laboratory of the Boston City Hospital, Boston, Mass.)

It was suggested in 1921 by Mallory, Parker and Nye [1] that hemochromatosis might be due to chronic poisoning with copper. The idea was based on experimental work which showed that pigment cirrhosis could be produced in rabbits and sheep by adding copper to their food over a long period of time.

The suggestion was urged again by Mallory [2, 3] in 1925 and 1926 on the basis of further experimental work and of the study of ten new cases of hemochromatosis which had come to postmortem examination within one year at the Boston City Hospital. Particular attention was paid to indulgence in alcoholic beverages and to occupations. Examination of a number of bootleg liquors for copper demonstrated it to be present in varying amounts and occasionally in considerable quantity in about 10 per cent of the samples tested. Occupations involving exposure to inhalation or ingestion of metallic copper or of its salts was shown to play a possible part in causing chronic poisoning in a certain number of instances. One man had worked for fourteen years in a shop "milling and planing copper and brass."

One of the conclusions drawn in the first paper published was the following: "Proof that hemochromatosis is due to poisoning with copper would require the demonstration of copper either in hemofuscin, or in the liver in excess of the minute amount said to be normally present, or in excretions from the body."

It is believed that all three of these requirements have been fulfilled by the work described in this paper.

The discovery by Mallory and Parker that it was easily possible by means of two delicate microchemical methods to demonstrate copper in sections of the livers of rabbits and other animals acutely

* Presented April 3, 1931, before the American Association of Pathologists and Bacteriologists at Cleveland, Ohio.
Received for publication May 27, 1931.

poisoned with copper led to the application of the same methods to the livers from human cases of pigment cirrhosis. The hematoxylin test was found to be much the more useful. Certain points must, however, be borne in mind in the application of the method. Copper is easily removed from tissues by the action of acids and alkalies even if they are very dilute. On this account alcohol would seem to be the best fixative. But after alcohol fixation hematoxylin stains hemosiderin brownish black to black while the copper in the pigment granules is colored light to dark blue. As a result the two kinds of pigment granules may be stained so much alike that they cannot always be distinguished from each other with certainty.

After fixation in formalin or in Kaiserling I, hematoxylin stains hemosiderin yellow to brown (brownish black in the macrophages) while the copper is colored blue, as after alcohol fixation. The reducing action of formalin therefore renders possible a clear differentiation of the two metals. The difficulty is to prevent the disappearance of the copper owing to the acidity which always develops in formalin. Perhaps the best method is to fix in as neutral formalin as possible for 1 to 3 days, wash in running water for 24 hours and preserve in 80 per cent alcohol. Probably neutral buffered formalin could be used to advantage but this method has not yet been tried.

Fortunately copper can often be demonstrated, at least in the coarser pigment granules containing it, even after the tissues have been preserved in formalin for a long time, although it cannot always be stained so intensely as could be desired.

The hematoxylin staining method has already been given in detail. It may be repeated briefly as it should be applied to pigment cirrhosis material obtained in the future.

1. Fix thin slices of liver tissue in neutral buffered (pH 7.0) 10 per cent formalin for 1 to 3 days. Wash in running water for 24 hours. Preserve in 80 per cent alcohol.

2. Stain frozen, celloidin or paraffin sections in a freshly prepared neutral buffered (pH 7.0) approximately 0.5 per cent aqueous solution of hematoxylin for about 1 hour.

3. Wash in several changes of tap water and then allow to stand in it for 1 hour in order to render the blue color of the copper brighter.

4. Alcohol, oil of origanum (cretic) or xylol, xylol balsam.

Copper in the pigment granules and in inspissated bile is stained

light to dark blue; iron (hemosiderin) yellowish brown to brownish black.

In examining pigment cirrhosis livers for the presence of copper it is necessary to select the most active cases because otherwise all the copper may have disappeared. What is wanted are livers which contain numerous islands of regeneration, because it is only in young liver cells just pigmented that copper can be found. We have studied five livers which fulfil these conditions and found copper present in all of them.

The best preservation of copper was in the most recent case, probably because the acid in the formalin had had little time to dissolve out the metal, although it may have been due to the fact that at times we have attempted to neutralize the fixative. One of the five cases dated back to 1917 and it was still possible to demonstrate copper in the coarser pigment granules. In two of the more recent cases it was impossible to demonstrate copper after formalin fixation, probably owing to marked acidity, but easy after alcohol fixation.

The findings in the five different cases of pigment cirrhosis were much alike so that they may be considered together. The islands of regeneration were of all sizes, from a few cells up to areas 2 and 3 mm. in diameter and of various ages. Some contained no pigment granules, others many, both fine and coarse. Practically all of the regenerating foci had a distinct bluish tint, suggesting that copper was present in the cytoplasm of the liver cells as well as in the pigment granules. As the cells in the foci aged and the copper was dissolved out the pigment granules either appeared yellow, taking no stain from the hematoxylin, or showed all gradations from blue to black owing to the development of hemosiderin while more or less copper was still present. The presence of the iron pigment could be demonstrated also by means of the ferrocyanide of potassium and hydrochloric acid reaction.

In a few of the islands of regeneration and less often elsewhere small masses of inspissated bile were present owing to obstruction to its outflow. These masses stained light to dark blue as a result evidently of the presence of copper in them because ordinary inspissated bile is not stained by hematoxylin.

These five cases of pigment cirrhosis demonstrate therefore beyond question, as the result of the differential hematoxylin stain, that

copper is present in the islands of regeneration. Moreover it was possible by means of the triple nitrite method to verify these results in two out of the four cases tested, as it showed copper in the sections, although not its exact location, while sections of control normal livers failed to do so.

Discussion

Owing in part at least to our suggestion ten years ago that hemochromatosis might be due to chronic poisoning with copper, much chemical work has been done during the past three or four years by Schönheimer and his coworkers on the question of an increase above the normal in the amount of copper in the liver in pigment cirrhosis.

In 1928 Schönheimer and Oshima [4] reported the examination of livers from seventeen cases of hemochromatosis and stated that they had demonstrated an average increase in the amount of copper of three to four times the normal. Herkel,[5] continuing their work, reported the examination of twenty-four additional cases. He found up to ten times the normal amount of copper. However, he examined also ten cases of non-pigmented cirrhosis. Two contained normal amounts of copper but the other eight showed a marked increase, in part exceeding that present in pigment cirrhosis. As a result of his chemical examinations he did not feel that he was justified in holding copper responsible for causing hemochromatosis.

By means of two very delicate chemical tests we have been able to demonstrate copper in the islands of regeneration in five cases of active pigment cirrhosis. It occurs in the pigment granules of the young regenerating liver cells and also in the masses of inspissated bile occasionally present as the result of focal bile stasis.

The study of the lesions in these five cases demonstrates clearly that copper is deposited in the young liver cells and is bound up with a derivative of hemoglobin with which it forms yellow pigment granules (copper hemofuscin). In the course of weeks to months the copper disappears, apparently in the bile in which it is regularly present in demonstrable quantity, and leaves behind pigment granules which for a time may give no reaction for iron and are called hemofuscin. Both types of hemofuscin granules stain deeply with basic aniline dyes. Later these pigment granules undergo a chemical change as a result of which they react for iron (hemosiderin). Some-

times this change takes place rather quickly while more or less copper is still present in the granules.

The quick elimination of the copper is supported by our work on acute copper poisoning in rabbits and other animals in which we showed that the copper had practically disappeared from the liver within five months after subcutaneous administration of it had ceased.

The fact that so much more iron than copper is present in the liver in hemochromatosis is difficult at first to understand. From the evidence presented the explanation is probably simple: the copper is quickly and steadily eliminated in the bile. The iron in the hemoglobin derivatives remains behind and accumulates for years. Apparently it cannot be eliminated until hemofuscin breaks down to hemosiderin. Then it very slowly dissolves and disappears. The injurious action of the copper is in part due to its combining with hemoglobin and causing it to be deposited as copper hemofuscin in the liver and other organs.

The presence of considerable copper in non-pigmented cirrhoses may be due to the retention in the bile of the copper normally eliminated in it. Focal bile stasis as the result of cirrhosis would lead to the accumulation of the normal amount of copper passing through the liver.

Owing to the ready solubility of copper in acids and alkalies it seems evident that chemists will not obtain the correct amount of this metal present in livers in hemochromatosis until they use dried fresh material or preserved material plus all the fluid in which it was fixed.

The presence of copper in the inspissated bile in these cases of pigment cirrhosis is of interest in view of the recent work by Schönheimer and Herkel.[6] They have shown that pigment gall-stones contain copper in large amounts up to 10,000 mg. per kilo, far more than is present in any organ or tissue in the body. Their observations and ours indicate clearly that copper is eliminated through the bile and therefore through the gastro-intestinal tract.

The cause of necrosis of the liver cells which finally leads to the formation of pigment cirrhosis has always been a puzzle. An equal amount of pigment in the liver cells in pernicious anemia produces no such effect. The demonstration that copper occurs focally in con-

siderable amount in the liver in pigment cirrhosis reasonably suggests that it is the cause of the lesion, because it has been shown that this metal when introduced into rabbits and other animals in sufficient quantity leads to marked pigmentation and to necrosis of liver cells, terminating in cirrhosis. In favor of this view is the fact that necrosis is more marked centrally, pigmentation peripherally in the lobule.

Summary and Conclusions

1. The presence of copper was demonstrated in islands of regeneration in five active cases of pigment cirrhosis by means of the hematoxylin test. Its presence in the sections was confirmed by the triple nitrite test in two of the four cases tested.

2. The copper occurred in pigment granules in the young liver cells and in masses of inspissated bile.

3. After causing the deposition of a copper hemoglobin compound the copper is quickly eliminated in the bile and therefore does not accumulate in the liver.

4. The pigments (copper-hemofuscin, hemofuscin and hemosiderin), derived successively from hemoglobin and containing masked and unmasked iron, require years to transform them. As a result they accumulate in large amounts in the liver and form the most prominent feature of this type of cirrhosis.

5. The necrosis of liver cells, which eventually results in cirrhosis, is apparently due to the toxic action of copper and not to the mechanical presence of the pigments.

6. Proof has been presented that copper is present in the early hemofuscin pigment granules, in the excretion, bile, and also in excess of normal in the liver tissue, as evidence that chronic poisoning with copper causes hemochromatosis.

REFERENCES

1. Mallory, F. B., Parker, Frederic, Jr., and Nye, Robert N. Experimental pigment cirrhosis due to copper and its relation to hemochromatosis. *J. Med. Res.*, 1921, **42**, 461.

2. Mallory, F. B. The relation of chronic poisoning with copper to hemochromatosis. *Am. J. Path.*, 1925, **1**, 117.

3. Mallory, F. B. Hemochromatosis and chronic poisoning with copper. *Arch. Int. Med.*, 1926, **37**, 336.

4. Schönheimer, R., and Oshima, F. Der Kupfergehalt normaler und pathologischer Organe. *Ztschr. f. physiol. Chem.*, 1929, **180**, 249.

5. Herkel, W. Über die Bedeutung des Kupfers (Zinks und Mangans) in der Biologie und Pathologie. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1930, **85**, 513.

6. Schönheimer, R., and Herkel, W. Uber das Vorkommen von Schwermetallen in Menschlichen Gallensteinen. *Klin. Wchnschr.*, 1931, **10**, 345.

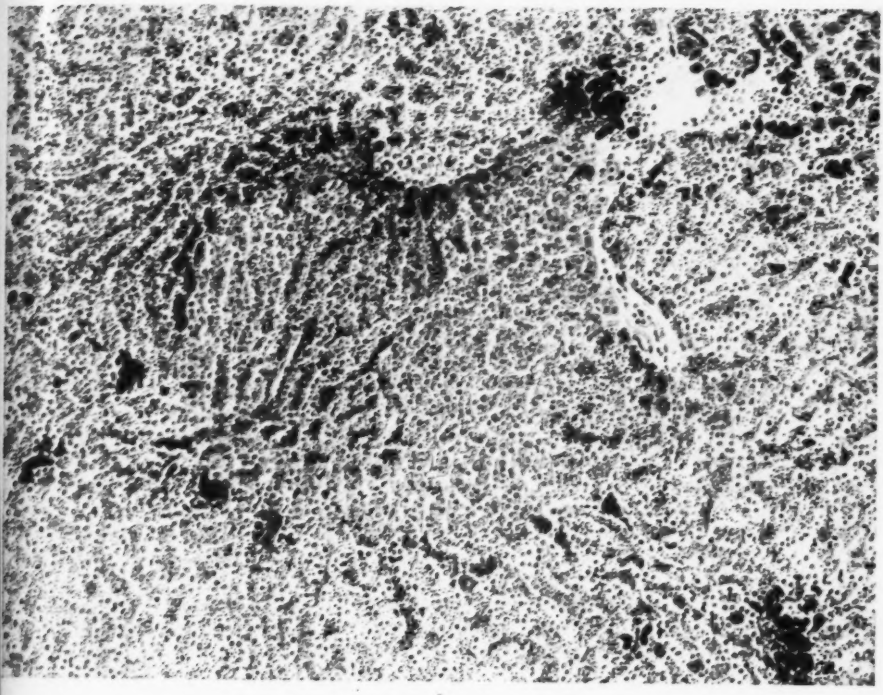
DESCRIPTION OF PLATE

PLATE 66

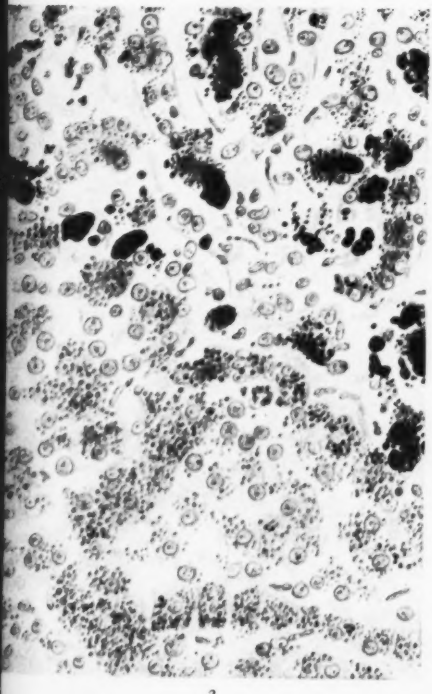
FIG. 1. Pigment cirrhosis (S 31–218). Kaiserling fixation. Section stained for 1 hour in neutral aqueous solution of hematoxylin. Pigment granules in liver cells in an area of regeneration colored blue, hemosiderin granules in adjoining liver cells and macrophages yellowish brown to black.

FIG. 2. Pigment cirrhosis (S 31–218). Fixation and staining as in Fig. 1. Pigment granules in regenerated liver cells and in masses of inspissated bile colored deep blue.

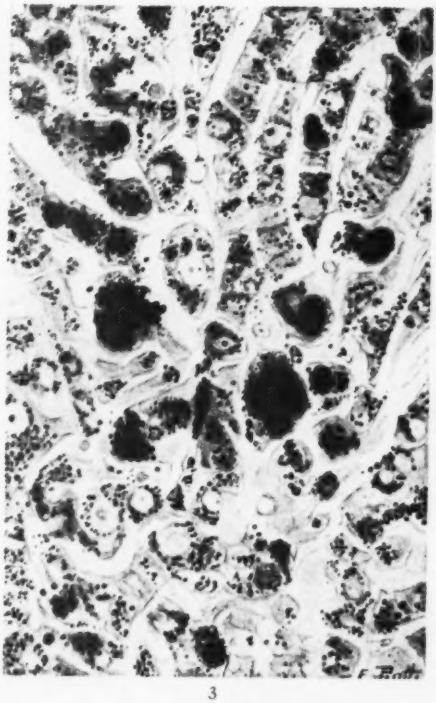
FIG. 3. Liver of rabbit (Z 690) injected subcutaneously with 2 cc. of a 20 per cent suspension of powdered metallic copper in lard and killed at the end of fourteen days. Pigment granules in liver cells and macrophages stained deep blue in 1 hour in a neutral aqueous solution of hematoxylin.



1



2



3

Mallory and Parker

Demonstration of Copper in Pigment Cirrhosis

SPONTANEOUS AND EXPERIMENTAL INFECTION OF PIGEONS WITH B. AERTRYCKE *

J. R. Cash, M.D., and C. A. Doan, M.D.
(From the Laboratories of the Rockefeller Institute for Medical Research, New York, N. Y., and the Department of Pathology of the Peiping Union Medical College, Peiping, China)

Introduction

During the course of some experiments upon blood formation in pigeons, which had first been undernourished for varying periods of time and then maintained upon diets consisting mainly of fetal beef tissues, an occasional bird became critically ill, developing diarrhea, grave anemia, and high leucocytosis with many young myeloid cells in the peripheral blood. Death invariably occurred within a few days after the onset of these symptoms. Such pigeons, though greatly emaciated, showed at autopsy marked enlargement of the liver, spleen and kidneys, as well as extreme myeloid hyperplasia of the bone marrow. Throughout the liver and kidneys, innumerable small yellowish gray points and irregularly shaped zones were grossly visible. Upon microscopic examination these areas were found to consist mainly of large numbers of myelocytes grouped about the blood vessels in these organs. The spleen showed a marked increase of large, clear, mononuclear, phagocytic cells and almost complete absence of lymphoid tissue, but contained no such accumulations of myelocytes as were seen in the liver and kidneys.

These changes presented a striking contrast to the fatty bone marrow and atrophied organs of the other equally poorly nourished pigeons of our series dying or killed after identical experimental procedures. Though it appeared that the exceptional pigeons were dying of some unusual complication, possibly of bacterial origin, we were unfamiliar with any variety of microörganism which had been shown to be capable of producing such lesions. Therefore, the changes in the blood and bone marrow, as well as the extensive extramedullary formation of myeloid tissue, seemed to us to present a problem worthy of further study.

* Received for publication March 27, 1931.

Preliminary Experiments

No bacteriological studies were made upon the first three birds exhibiting the unusual features just mentioned, but cultures of the blood, liver, spleen, kidney and bone marrow of the fourth bird (Pigeon 86) to die under these conditions all gave pure growths of a small bacillus which was identified by Dr. L. T. Webster of the Rockefeller Institute as *Bacterium aertrycke*.

This organism, which varies from 1 to 2.5 microns in length and from 0.3 to 0.4 of a micron in thickness, is a Gram-negative, motile rod with rounded ends. In smears prepared from young cultures, the bacillus varies in morphology from rod to almost coccoid forms and its body stains evenly, though individual organisms with more deeply staining ends are usually present. Numerous long, peritrichal flagella are revealed by appropriate stains. In cultures, both acid and gas are produced in dextrose, maltose, mannite, levulose, xylose, galactose and dulcite, the reactions being more marked in the last four sugars than in the three former ones. Saccharose and lactose are not affected, even after several days' incubation. Indol is not formed. Litmus milk is rendered slightly acid within the first twenty-four hours but the reaction later changes to alkaline. Milk is not clotted. On potato the growth is very poor, forming only a faintly visible, grayish white pellicle. Coagulated serum and gelatin are not liquefied. In broth the growth produces even cloudiness, but after several days a grayish white sediment is usually formed. Cultures on lead acetate medium show that H_2S is produced. On meat infusion agar slant the organism forms a thin, transparent, confluent growth, a characteristic of all bacteria belonging to the paratyphoid group. On blood agar plates the growth is luxuriant; large, round, flat colonies with pale centers are formed in twenty-four hours. On China blue rosolic acid medium the colonies are small, round, slightly elevated and of distinctly pinkish color.

The immunological behavior of this organism strongly suggests that it represents a separate species. Cultures are agglutinated by immune pigeon and rabbit sera in high dilutions, the usual titre being 1:2560. *B. typhosus* and *B. paratyphosus B* are agglutinated by anti-aertrycke serum in dilutions of 1:40 and 1:10 respectively. Rabbit sera rendered immune to *B. typhosus*, *B. paratyphosus A*, *B*, and *C* usually agglutinate *B. aertrycke* in dilutions up to 1:40. The

blood of normal pigeons rarely contains agglutinins for *B. aertrycke*, and even on these occasions the bacilli are not agglutinated in dilutions greater than 1: 20.

Inasmuch as we have subsequently used the culture of organisms isolated from the liver of Pigeon 86 to produce all experimental infections, we submit the following complete protocol of this bird.

TABLE I

Protocol of Pigeon 86

Diet	Full grain (Apr. 24–26)	Starvation (Apr. 27–May 3)	5 gm. fetal liver daily (May 4–16)					Food refused	
Dates blood examined. . . .	April 25	May 2	May 6	7	10	13	14	15	16
White blood cells (thousands per cmm.)	14	4	11	9	14	55	69	133	187
Granulocytes %	40	54	78	86	64	87	88	91	85
Myelocytes %	0	0	0	0	0	0	0	0	5
Lymphocytes %	42	44	12	4	26	1	5	4	4
Monocytes %	18	2	10	10	10	12	7	5	6
Red blood cells (millions per cmm.)	3.7	2.6	3.3	3.1	3.1	2.5	2.2	1.9	1.2
Thrombocytes (thousands per cmm.)	75	16	25	36	37	26	59	33	37
Weight (gm.)	425	355	320	320	300	280	265	245	230 Died

The *liver* and *kidneys* were found to be greatly swollen and studded with massive accumulations of myelocytes intermingled with smaller, basophilic, poorly differentiated, mononuclear cells. Many large mononuclear phagocytes and bacteria were found in the sinuses of the liver. The *spleen* was also much enlarged, due mainly to the presence of large, mononuclear phagocytes, but showed no such accumulations of myeloid cells as were seen in the liver and kidneys. The heart, lungs, alimentary tract, reproductive organs and brain were unaltered.

At the time of autopsy the blood as well as emulsions of the various organs of Pigeon 86 were injected into a series of normal birds. Of three pigeons, each injected intraperitoneally with 1 cc. of blood, none developed any symptoms of disease. When these birds were killed for study a month later, no anatomical changes were found, but *B. aertrycke* was grown from the livers of two of them. Of seven

pigeons injected intraperitoneally with 2 cc. of a thick saline emulsion of liver, kidney, or bone marrow, two which had received liver became acutely ill within twenty-four hours and died forty-eight hours and five days respectively after inoculation. Shortly before death one of these birds exhibited a leucocytosis of 75,000 cells per cmm., of which 3 per cent were myelocytes. The other showed only a slight increase in total number of leucocytes, 21,000 per cmm., but of this number 71 per cent were myelocytes. These two pigeons showed acute fibrinopurulent peritonitis, as well as the same types of anatomical changes seen in Pigeon 86, and *B. aertrycke* was recovered from the liver of each of them in pure culture. None of the remaining five birds showed any symptoms of disease following injection, or any macroscopic lesions* when killed a month later, but *B. aertrycke* was grown from the livers of three of them at this time. Cultures made from the livers of the other two birds were sterile.

Three normal pigeons were injected intraperitoneally with 0.1 cc., 0.5 cc., and 1 cc. respectively of a pure 24 hour broth culture of *B. aertrycke*, originally isolated from the liver of Pigeon 86. The first bird showed no manifestations of disease and no anatomical changes when killed a month later. The other two birds, however, died four days and thirty-six hours respectively after inoculation, showing all of the clinical and most of the anatomical characteristics of the disease under study. In addition, fibrinopurulent peritonitis was present in both instances. No myelocytic infiltrations were found in the liver and kidneys of the bird dying thirty-six hours after inoculation. From the livers of all three pigeons *B. aertrycke* was isolated in pure culture.

It therefore seemed highly probable that infection with *B. aertrycke* was the cause of the unusual lesions and death of certain of our birds but, inasmuch as each pigeon was kept in a separate cage and cultures of their water and food failed to reveal the presence of this organism, the source of the infection was not clear. However, since it had been demonstrated experimentally that small doses of *B. aertrycke* had little or no effect upon normal pigeons, and, moreover, that these bacteria often survived within the body for at least a month without producing symptoms of disease or anatomical

* In the removal of this work from New York to Peiping the microscopic preparations made from the tissues of these birds were unfortunately lost.

changes, it did not appear unlikely that certain pigeons naturally harbored minimal numbers of this organism and developed generalized infection when subjected to a state of malnutrition. At this point it is of interest to note that a single previously normal pigeon (No. 94), after a period of eight days' starvation, became unduly ill, totally refused food and died within forty-eight hours, showing all of the lesions accompanying infection with *B. aertrycke*. This organism was recovered in pure culture from the liver.

In order to determine whether or not pigeons dying of malnutrition, but showing no evidence of bacterial infection, ever harbored *B. aertrycke* in their tissues, cultures were made of the livers of twenty such birds which had been kept under experimental conditions identical with those of the pigeons dying of infection with this organism. Sixteen of these cultures showed no growth of bacteria, but from four birds *B. aertrycke* was recovered. Though none of these four pigeons showed changes in the blood before death which would suggest infection with *B. aertrycke*, in two of them the characteristic lesions accompanying infection with this organism were found. The other two showed only the effects of malnutrition.

The results of these preliminary studies may be summarized as follows: In a series of fifty-two pigeons, which first had been subjected to a period of starvation and then maintained for varying periods of time on fetal beef tissues, seven cases of an acute, fatal disease associated with marked anemia and enormous activity of myeloid cells occurred. From three of these birds, which were studied bacteriologically, *B. aertrycke* was isolated in pure culture, and in the tissues of the other four an abundance of bacteria morphologically identical with this organism were demonstrated. Four additional instances of the presence of *B. aertrycke* were encountered in twenty routine bacteriological examinations of pigeons of this series showing no clinical evidence of bacterial infection. Two of these showed anatomical changes attributable to the infection with *B. aertrycke*, while the other two did not.

Although it was demonstrated that a disease process could be initiated in normal pigeons by inoculation of tissue from an infected bird as well as by inoculation of cultures of *B. aertrycke*, the nature of the lesions, in both the spontaneous and experimental infections, caused us to have some doubt as to their exclusive relationship to this organism. The lesions in which the bacteria were found,

consisting of aggregations of mononuclear phagocytes and polymorphonuclear leucocytes, were readily explained. However, such massive myeloid hyperplasia of the bone marrow, the frequent appearance of large numbers of myelocytes in the peripheral blood, and the striking heterotopic formation of myeloid tissue in the liver and kidneys presented a picture of myeloid activity hitherto unrecorded in the course of bacterial infections. Furthermore, since *B. aertrycke*, in several instances, had been found in pigeons which showed no symptoms or anatomical evidence of active infection with this organism, and also had been shown experimentally to survive in the tissues of pigeons for considerable periods of time without producing disease, it was obvious that further facts must be sought before the relationship of this organism and the interesting lesions with which it was frequently associated in pigeons could be understood.

Methods

Each bird, upon entering the laboratory, was placed in a separate cage and observed for a period of two weeks, during which time the weight was followed and the blood examined several times. Only those pigeons which appeared normal during this time were used for experimentation.

Supravital staining with neutral red and Janus green,[1] which facilitates a ready distinction of all the cellular elements of the blood, was used routinely in making the differential cell counts. Striking changes in the blood, such as the appearance of numerous young myeloid forms, were always checked in fixed films. The total counts of the red and white cells were made according to the method of Forkner,[2] which we have found to be thoroughly reliable.

In order that experimental infection might be more nearly comparable to infection naturally acquired, we have always introduced the bacteria by mouth, slowly feeding 1 cc. of a 24 hour pure broth culture, drop by drop, with a small pipette. After infection, the blood was studied at frequent intervals until death or recovery. Autopsies, as well as bacteriological and serological studies, were done promptly after death, an occasional moribund bird being killed in order to secure perfectly fresh tissues.

To test our cultures for the presence of some filterable form of microörganism or other substance which might have been respon-

sible for the excessive growth of myeloid tissue regularly accompanying infection with *B. aertrycke*, normal pigeons were fed or infected with sterile filtrates prepared from this organism.

To serve as controls for our other observations, bacteriological, serological and anatomical studies were made upon a series of apparently normal pigeons.

The Blood of Normal Pigeons

The blood of normal pigeons has been the object of study of only a few investigators,[3, 4, 5] but all of those who have dealt with it have reported marked fluctuations in number of all types of blood cells.

During the course of our experience, based upon 182 apparently healthy pigeons bought at random in the markets of New York and Peiping, we have found that the red blood cells remain unusually constant in individual birds, fluctuations of as much as 400,000 cells being quite exceptional. When first brought into the laboratory, most pigeons show erythrocyte counts of 3,500,000 to 4,000,000 cells per cmm. Only an occasional bird shows a slightly higher count, while erythrocyte counts lower than 3,000,000 almost invariably have been found to be associated with some obvious disease.

The white blood cells, however, have shown considerably greater fluctuations in number than have the erythrocytes. Though the total number of white cells was generally found to lie between 8,000 and 20,000 per cmm., an occasional bird, in which no disease was apparent, was studied which presented a count far above or below these levels. We have seen a few such pigeons with leucocyte counts of 50,000 cells per cmm., for which there was no obvious explanation. However, after being kept in the laboratory for a few days, the white cells in such instances have invariably fallen to normal levels and later on have shown no tendency to rise to their original height.

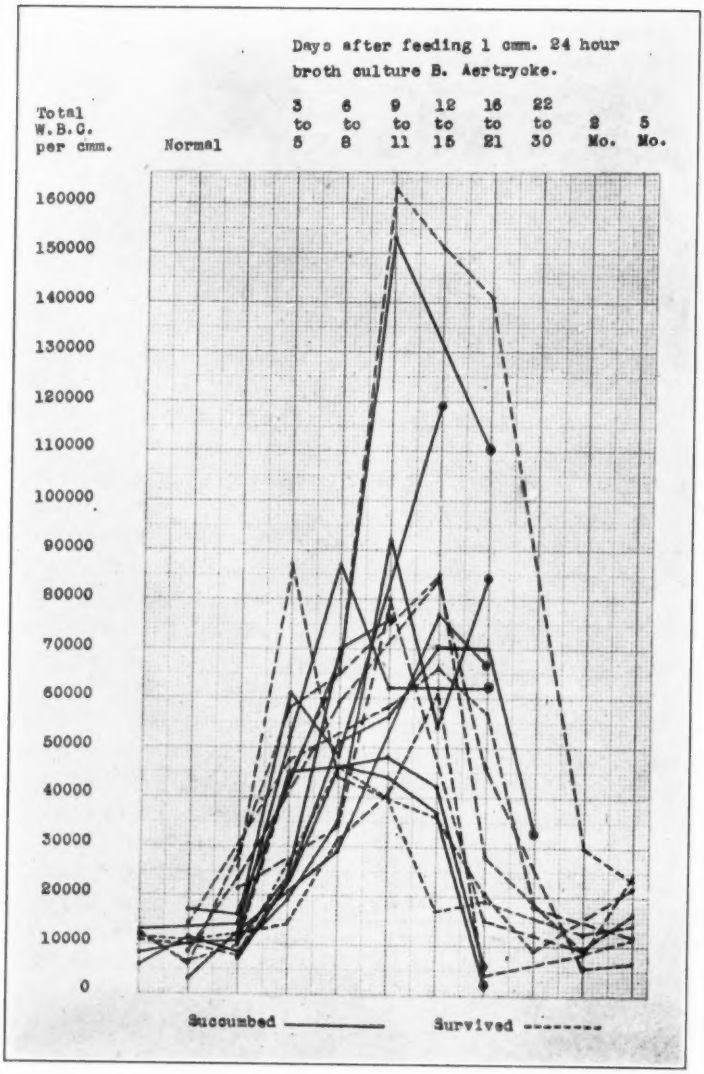
The percentages of the different varieties of blood cells also have been found to vary greatly in different birds. They have remained quite constant in individuals, though the total white cell counts of these same pigeons have varied as much as 10,000 cells from day to day. White counts made in rapid succession upon the same bird almost always agree closely.

The morphological characteristics of the various elements of the pigeon's blood, as far as we know, are nowhere adequately illus-

trated, though Forkner[2] has recently presented a well illustrated study of the blood of normal fowls, which in most essential features resembles that of the pigeon. We have, therefore, devoted most of the accompanying Plate 67, which illustrates supravitally stained cells from the blood of pigeons, to the early myelocytic types encountered in the circulation in our experimental birds.

The granules of the main group of polymorphonuclear leucocytes (Figs. 1 and 2) of the pigeon, as in the fowl, are rod-shaped but exhibit a decidedly more brilliant and darker yellowish red color when stained either supravitally with neutral red or in fixed films with Wright's stain. We have found no cells in the pigeon's blood closely corresponding to the "pseudoeosinophils" found in small numbers in the fowl's blood by Forkner, but we have observed regularly a few myeloid cells, not present in the blood of fowls, with pleomorphic or round nuclei and large, brilliantly eosinophilic granules in their cytoplasm. Such cells, which generally exist only in small numbers, less than 5 per cent, are found to be much more numerous in occasional birds, sometimes comprising 50 per cent of the total number of leucocytes. The relationship of these cells to the other cells of the myeloid series is not clear. They apparently tend to remain high in individual birds which show no other abnormality but, if for any reason a leucocytosis occurs, their numbers have never been observed to increase. We have looked upon such cells as true eosinophils and at first thought that their large numbers were perhaps associated with parasitic infections. Though this association has existed in a few instances, such is frequently not the case. Only recently we have seen an apparently normal pigeon with the duodenum and ileum greatly distended with a solid mass of small nematodes in which the total white cell count was 15,000 per cmm., of which only 2 per cent were eosinophilic myeloid cells with round granules. The granules of the basophils of the pigeon are very small, round and stain deep crimson with neutral red. The monocytes (Figs. 12, 13), lymphocytes (Fig. 14), erythrocytes, and thrombocytes show only very slight variations in morphology from those of the fowl. The general appearance of the myelocytes of the two species is about the same, though slight differences in color of the granules exist in both supravitally stained (Figs. 5 to 11) and fixed preparations.

In the following table are summarized the results of our studies


Days after feeding 1 cmm. 24 hour broth culture B. Aertrycke.
Total W.B.C. per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2 Mo.
5 Mo.
160000
150000
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Succumbed
Survived

of the blood of normal pigeons. Only birds which were apparently free from disease have been included. The figures given represent average values.

TABLE II

Blood Counts on Normal Pigeons

	New York	Peiping	Total
Number of Pigeons	100	82	182
Number of Blood Counts	238	139	377

	New York		Peiping	
	Total cells per cmm.	Percentage	Total cells per cmm.	Percentage
Erythrocytes	3,813,000	..	3,803,000	..
Leucocytes	13,100	..	15,200	..
Thromboyctes	34,060	..	42,520	..
Eosinophilic leucocytes with rods	5,371	41	5,320	35
Eosinophilic leucocytes with round granules	917	7	760	5
Basophilic leucocytes	393	3	304	2
Lymphocytes	5,109	39	7,144	47
Monocytes	1,310	10	1,672	11

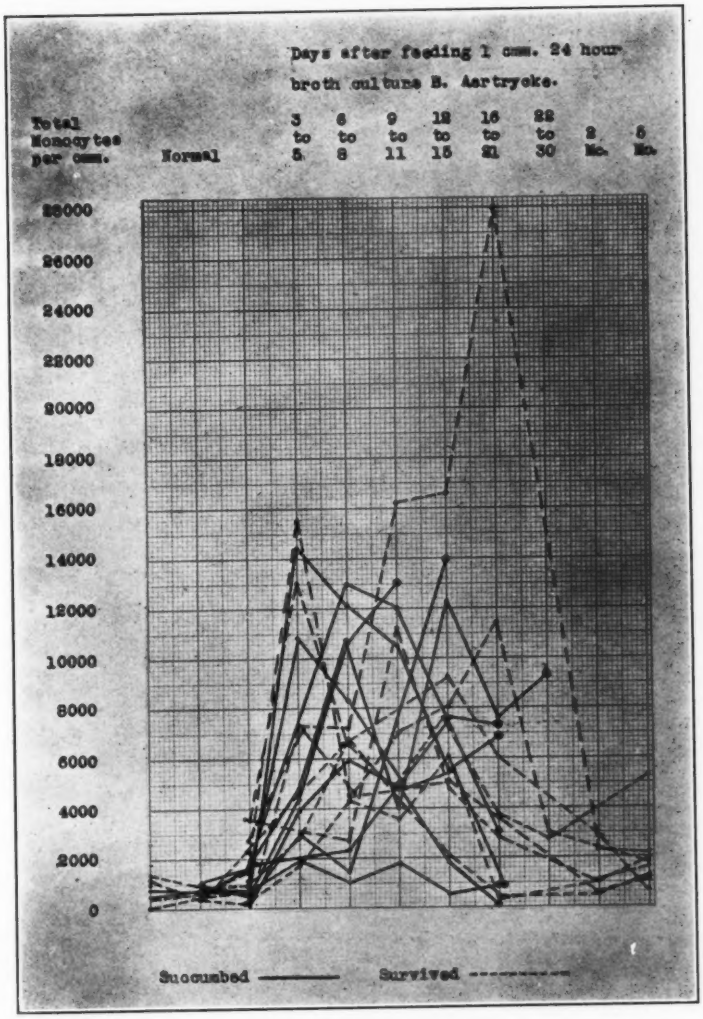
THE EXPERIMENTAL INFECTION OF PIGEONS WITH B. AERTRYCKE

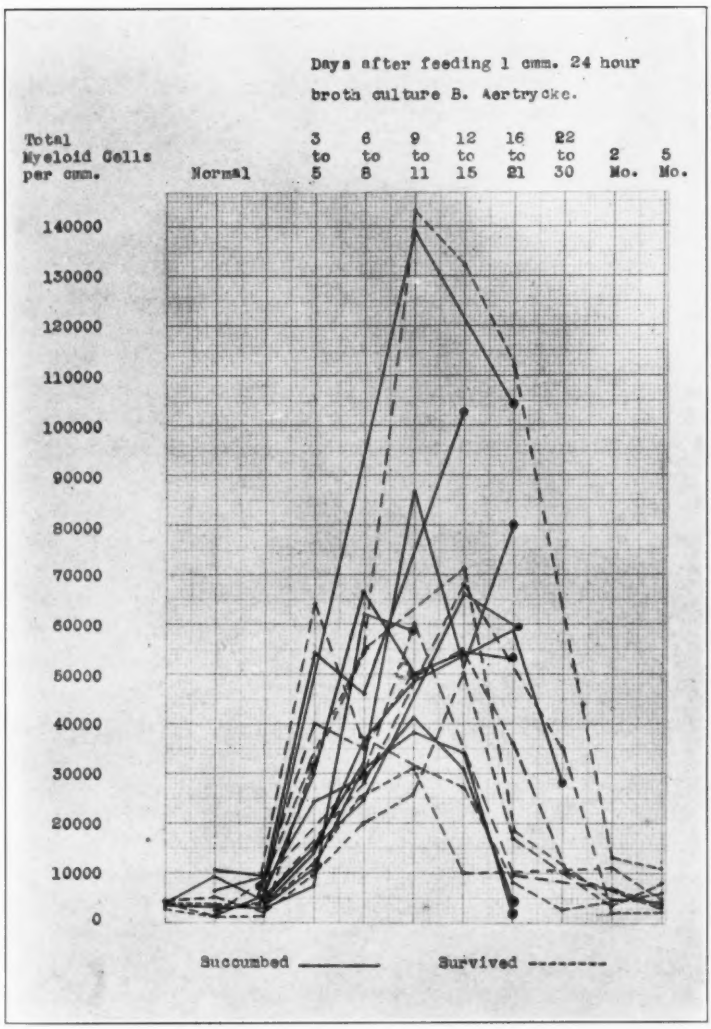
After two weeks preliminary observation, during which time their blood was studied, seventeen apparently normal pigeons were each fed 1 cc. of a 24 hour broth culture of *B. aertrycke*. All of these pigeons became acutely ill within thirty-six hours, most of them developing diarrhea and showing loss of weight, although given food and water in unlimited amounts. Nine of them died within ten to twenty-five days after infection; the remaining eight recovered.

Changes in the Blood: After feeding *B. aertrycke* the total number of leucocytes, which had varied in different birds under normal conditions from 6,000 to 29,000 cells per cmm., rose sharply to levels of 46,000 to 160,000 cells per cmm. (Chart 1). Most of the pigeons which died succumbed at a point near the peak of their leucocytosis, but in two birds, after only moderately high leucocytoses of 46,000 and 48,000 cells per cmm., the total number of white cells suddenly fell to 2,000 and 6,000 cells per cmm. respectively. Examination of the bone marrow of these two birds after death showed very few myeloid forms but did reveal a striking degree of hyperplasia of the erythrogenic cells. Extensive myeloid infiltration was present, however, in kidneys and liver of each bird. This particular observation

is suggestive of the two types of leukemic manifestation, erythroleucosis and myeloid leucosis, reported as occurring in different fowls inoculated with identical material from an animal dying of a typical myeloid leukemia. That is, with apparently the same underlying stimulus, two different responses may be elicited, depending upon the individual bird. It is not known at present what the nature of the factors governing the individual response may be.

The leucocytosis following infection with *B. aertrycke* was clearly due primarily to an increase in number of the eosinophilic polymorphonuclear leucocytes with rod-shaped granules (Chart 2). Within three to five days after infection mitochondria became visible in the motile granulocytes. The delicately shaped rods of these cells became greatly swollen (Figs. 1, 2, 4), assuming an oval or rounded form though they still stained brilliantly, and atypical microcytes (Fig. 3) were found. After a week many of these younger adult forms of the eosinophilic rod cells, as shown by their slightly indented nuclei and numerous mitochondria, were seen regularly in the peripheral blood. The motility of all granulocytes was always strikingly decreased during the entire course of the infection. Actual myelocytes, frequently very young forms, appeared in practically all pigeons of this group in numbers varying from 2 to 10 per cent of the total number of leucocytes, but in no instance of experimental infection have we observed such a great number of myelocytes in the peripheral blood as frequently occurred in undernourished pigeons dying from infection with *B. aertrycke* naturally acquired. Fig. 7 represents the late myelocytes "C," [32] with the complement of specific preleucocytic granules nearing the maximum. The myelocytes of the bird have spherical granules throughout the period of maturation in bone marrow, the change to rods occurring coincident with the change in staining reaction, lobing of the nucleus and the acquisition of motility, which immediately precede the extramedullary circulatory appearance and function of these cells. Under normal conditions only fully mature cells with none or very occasional mitochondria, and only rod-shaped granules appear in the circulation. Figs. 5, 8, and 10 represent the stage known as myelocyte "B," midway between myelocyte "C" and the earliest myeloid cells showing specific granulation, namely myelocytes "A" (Figs. 6, 9 and 11). No changes in the polymorphonuclear leucocytes with round eosinophilic granules or in the basophils were noted.


Days after feeding 1 cmm. 24 hour broth culture B. Aertrycke.
Total Monocytes per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2 Mo.
6 Mo.
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Succumbed
Survived


Days after feeding 1 cmm. 24 hour broth culture B. Aertrycke.
Total Myeloid Cells per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2 Mo.
5 Mo.
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Succumbed
Survived

The monocytes showed considerable increase in total number after infection with *B. aertrycke*, tending to reach their highest point before the fifteenth day. Many young monocytes with dense, homogeneous cytoplasm and numerous mitochondria appeared regularly during the infection (Chart 3).

The lymphocytes exhibited a general tendency to fall during the course of the infection. Especially in those birds which succumbed, the diminution of lymphocytes was most marked (Chart 4).

Changes in the Viscera: The changes observed in the viscera of pigeons following oral administration of a large dose of *B. aertrycke* have been almost identical in all birds dying as the result of infection. Therefore, since individual birds showed only minor variations in the degree of lesions commonly present, these changes will be discussed collectively. By far the most interesting are those of the liver, kidney, bone marrow and spleen.

The *liver* is always enlarged, at times reaching a weight of 20 gm. It has an opaque, swollen appearance, is generally of pale, brownish red color, and throughout the organ small, round or irregularly shaped patches of opaque, yellowish gray tissue are seen. Microscopically, the liver cells are found to be greatly swollen and the sinuses filled with large, pale, phagocytic cells containing cellular débris, hemosiderin and occasionally red blood cells. Such phagocytic cells, together with a few polymorphonuclear leucocytes, are often found in solid masses, showing varying degrees of necrosis in their central areas where clumps of small Gram-negative bacilli are usually present (Figs. 15, 16). Many such bacilli are scattered in smaller number throughout the liver sinuses. In addition to these lesions, which clearly seem to be due directly to the bacteria which are found within them, each portal and hepatic vein is found to be surrounded by varying numbers of myelocytes intermingled with lesser numbers of slightly smaller, basophilic, mononuclear cells, which contain no granules in their cytoplasm (Figs. 17, 18, 19). In early infections these latter cells have occasionally outnumbered the myelocytes with which they were associated. The myeloid tissue usually present is quite excessive, frequently forming areas visible to the naked eye. Bacteria have never been found in these lesions.

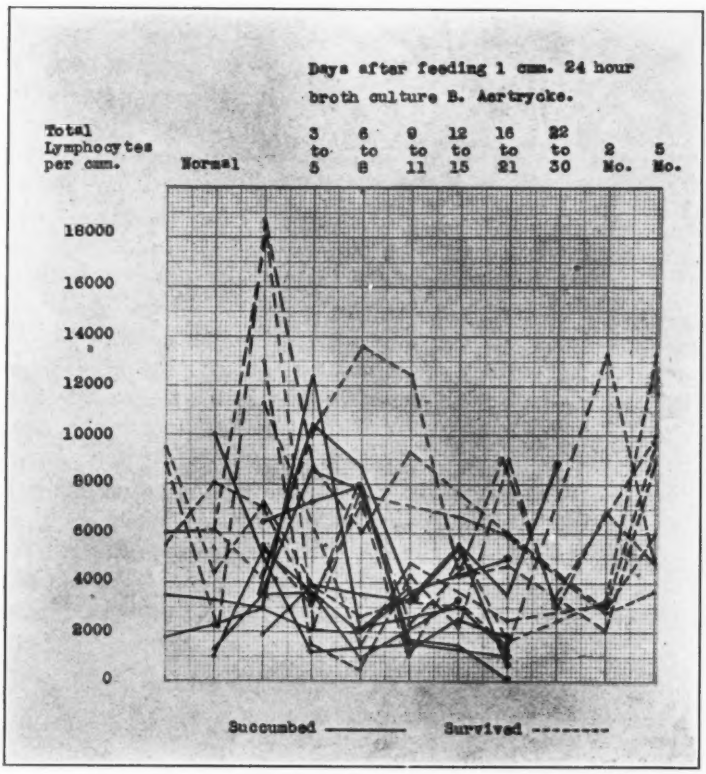
The *kidneys* are always considerably swollen and of pale, yellowish brown color. Large foci of myelocytes and basophilic, mononuclear cells apparently arising about the blood vessels, are regu-

larly present which closely correspond to the lesions seen in the liver (Fig. 20). In some instances there are also small collections of mononuclear, non-granular cells scattered throughout the interstitial tissue of the kidneys. The epithelium of the tubules is generally much swollen.

Of all the changes occurring in pigeons after infection with *B. aertrycke* those found in the *bone marrow* are perhaps the most extensive. The radius, ulna and femur, which were examined regularly, were filled with solid, firm masses of grayish red, opaque marrow, which literally burst forth from the cracks in the shaft during the process of removal. On microscopic examination one sees a solid mass of myeloid cells in which young forms predominate. The capillaries are collapsed by pressure of the proliferating myelocytes and erythrogenesis, so conspicuous in the marrow of normal pigeons, is seen only in certain very limited areas (Figs. 21, 22). Scattered throughout the marrow are found in many instances large and small aggregations of pale, mononuclear cells similar to those seen in the liver. Occasionally, bacteria may be demonstrated in these areas. Capillaries filled with fresh thrombi and bacteria are seen frequently.

The *spleen* is enlarged from one to three times its normal size, is of very soft consistency and pale pink or yellowish brown in color. Microscopically, the most striking alteration is found to be a marked proliferation of large, clear mononuclear cells. This change, which takes place in the follicles as well as the pulp, is attended with almost total disappearance from the entire spleen of cells which can be identified as lymphocytes. Within the sinuses and throughout the pulp many such phagocytic cells, frequently grouped in nodules with a few polymorphonuclear leucocytes about cellular débris and bacteria, are present. Though fairly numerous polymorphonuclear leucocytes and, in a few instances, occasional myelocytes are seen scattered throughout the spleen, no collections of myeloid tissue occur in the spleen which are in any way comparable to those seen regularly in the liver and kidneys. In the case of only one pigeon, which was shown to be ill with acute *B. aertrycke* infection when brought into the laboratory, were large areas of young myelocytes found in the spleen.

The *lungs*, in most instances where infection has been induced by mouth, have shown lobular pneumonia of an unusual type. Situated only in the dependent (anterior) portion of the lungs, about bronchi


Days after feeding 1 cmm. 24 hour broth culture B. Aertrycke.
Total Lymphocytes per cmm.
Normal
3 to 5
6 to 8
9 to 11
12 to 15
16 to 21
22 to 30
2 Mo.
5 Mo.
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Succumbed
Survived

plugged with necrotic exudate, were caseous, often coalescing areas of consolidation which were sharply demarcated from the surrounding tissue. Microscopically, such areas, within which were myriads of Gram-negative bacilli, were found to be composed of necrotic lung tissue and exudate well encapsulated by a thick zone of mononuclear phagocytes and polymorphonuclear leucocytes. *B. aertrycke* was recovered regularly from such patches of pneumonia. The constant, characteristic distribution of these lesions strongly suggests that they were caused by aspiration of a portion of the culture of bacilli with which the birds were fed. No lesions of any kind have occurred in the lungs of pigeons naturally infected with *B. aertrycke.*

We have never found any significant lesions in the *intestine* of pigeons following natural or experimental infection with *B. aertrycke.* Upon being introduced by mouth, these organisms may be recovered from the stool during the following three or four days in about 50 per cent of the birds. After this time, however, they tend to disappear from the intestine. Intestinal contents and mucosa have been cultured in twenty-six pigeons dying from five to twenty-five days after oral administration of *B. aertrycke,* but in only six instances was the organism recovered. Pure cultures of *B. aertrycke* were grown from the liver of each of these birds and morphologically identical organisms demonstrated in sections of other organs.

The *heart, pancreas, adrenals, thyroid, ovaries,* and *testes* have shown no lesions worthy of note. We have examined the *brain* in only four birds infected with *B. aertrycke,* but no lesions were found in these instances.

Experiments with Bacteria-Free Filtrates Prepared from Cultures of B. Aertrycke

Although the oral administration to pigeons of what seemed to be pure cultures of *B. aertrycke* was followed regularly by the disease which we have described, the deduction might not follow that all of the anatomical changes present were caused by this organism. It was clear that the lesions containing bacteria were the direct result of infection with *B. aertrycke,* but the possibility still existed that our cultures were contaminated with a filterable microörganism or contained some filterable agent which was responsible for the changes in the myeloid cells. Accordingly it was decided to test upon pigeons

the effect of bacteria-free filtrates prepared from virulent cultures of *B. aertrycke.*

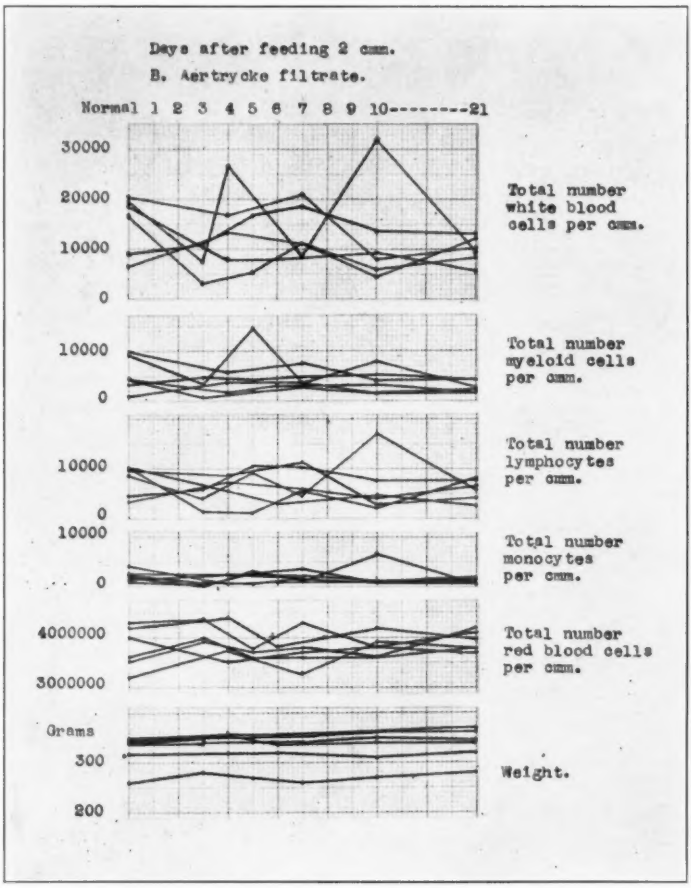
From a 200 cc. flask of a 24 hour broth culture of *B. aertrycke,* 100 cc. was passed through a VV Berkefeld filter. To test its sterility 40 cc. of this filtrate was immediately inoculated into a fresh flask of sterile broth, and this flask, together with the flask containing the remaining 60 cc. of filtrate, incubated overnight. No growth of bacteria occurred in either of these two flasks. Each of six apparently normal pigeons, whose blood had been found free of natural agglutinins to *B. aertrycke,* was then fed 2 cc. of this sterile *B. aertrycke* filtrate. To test the potency of the organism from which the filtrate was made, 1 cc. of the original broth culture of *B. aertrycke,* then 36 hours old, was fed to each of six other normal pigeons.

As shown in Chart 5, no appreciable changes occurred within twenty-one days in the blood of the six pigeons fed with filtrate, nor did they show any loss of weight or other evidence of disease. When they were killed at the end of this time, no agglutinins for *B. aertrycke* were found in their blood, cultures of liver were sterile in each instance, and no gross or microscopic lesions suggestive of infection with *B. aertrycke* were found in any of their tissues.

All of the six pigeons fed with the original culture of *B. aertrycke* from which the filtrate was made became acutely ill. Four died; two recovered. Autopsies upon the four which died showed all of the characteristic lesions of infection with *B. aertrycke* and this organism was recovered from the organs in each case. The sera of the two birds which recovered agglutinated cultures of *B. aertrycke* in dilutions of 1:320 and 1:2560 respectively one month after infection.

This experiment has since been repeated in a slightly different form. Another sample of filtrate, prepared as outlined above, was fed in amounts of 2 cc. to each of five normal pigeons and also injected intravenously in amounts varying from 0.2 to 1.5 cc. into each of five others. At the same time, as controls, two normal birds were injected intravenously with 1 and 2 cc. of sterile broth, respectively, and each of two others fed with 2 cc. of the same material. The virulence of the culture of *B. aertrycke* from which the filtrate was made was tested by feeding 1 cc. to each of two normal birds.

None of this series of ten pigeons, either fed or intravenously inoculated with *B. aertrycke* filtrate, or the four controls receiving


Days after feeding 2 cmm.
B. Aertrycke filtrate.
Normal 1 2 3 4 5 6 7 8 9 10--------21
30000
20000
10000
0
Total number white blood cells per cmm.
10000
0
Total number myeloid cells per cmm.
10000
0
Total number lymphocytes per cmm.
10000
0
Total number monocytes per cmm.
4000000
3000000
Total number red blood cells per cmm.
Grams
300
200
Weight.

sterile broth showed any change in blood picture, loss of weight or other evidence of disease during the ensuing month after administration of these substances. When killed at this time no gross or microscopic changes in any way resembling the lesions occurring in *B. aertrycke* infection were seen. Cultures of the liver made upon blood agar plates were universally sterile, and the blood in each case showed no agglutinins for *B. aertrycke*. Both of the controls, upon which the virulence of the culture of *B. aertrycke* was tested, died within ten days. They showed all of the characteristic symptoms and lesions produced by this organism, which was recovered from the livers of both birds.

Therefore, inasmuch as two different series of pigeons, in all numbering sixteen birds, either fed with or intravenously injected with large, single doses of broth filtrate prepared from virulent cultures of *B. aertrycke* have shown no clinical, anatomical, bacteriological or serological evidence of disease, it seems at least highly probable that all aspects of the disease-picture regularly occurring in pigeons after experimental infection with *B. aertrycke* are due entirely to that organism or its products. However, the possibility that a virus or other substance may be concerned in the alterations of the myeloid tissue is still to be kept in mind until the question has been studied further.

Studies upon Normal Pigeons

In order to gain some idea of the frequency with which pathological changes occur within the tissues of apparently normal pigeons, and to serve as a general control for our observations upon pigeons infected with *B. aertrycke*, we have studied sixteen birds which gave the appearance of being entirely free of disease. The results are summarized in Table III.

All of the six pigeons, which are stated to have been under observation for ten months, were kept in separate cages but in close proximity to the cages of pigeons infected with *B. aertrycke*. During this time their bloods were examined at frequent intervals but no significant changes were observed; none of them showed any loss of weight or other evidence of disease. The remaining twelve birds observed for one week before being killed for study were well nourished, took their food well, and their blood cells, upon two examinations, showed no pathological changes.

TABLE III

Summary of Pathological Changes in Sixteen Normal Pigeons used as Controls for Pigeons Infected with B. Aertrycke

No.	Period of observation	Cultures of liver	Agglutination *B. Aertrycke*	Pathological changes in tissues
7	10 months	No growth	1:64	Moderate increase in small, round cells about blood vessels of liver and kidney. In a few instances round masses of these cells resembling germinal centers of lymph glands seen
8	10 months	No growth	0	Three small groups of adult polymorphonuclear leucocytes with an occasional myelocyte among them seen adjacent to portal veins
10	10 months	No growth	0	Small round cells (lymphocytes?) slightly increased about vessels of liver
11	10 months	No growth	1:32	Enormous increase of lymphocytes about vessels of liver. Moderate increase of such cells in kidney
12	10 months	No growth	0	No lesions
13	10 months	No growth	0	No lesions
72	1 week	No growth	0	Very slight increase of round cells about vessels of liver
73	1 week	No growth	0	Considerable increase round cells in liver. Small healing necroses in liver and also small nodules consisting of central giant cells and débris surrounded by large, clear monocytic cells and lymphocytes. No tubercle bacilli demonstrable. These lesions do not resemble those of tuberculosis
75	1 week	No growth	0	No lesions
77	1 week	No growth	0	Liver shows nodules as in Pigeon 73. Moderate hyperplasia of bone marrow
79	1 week	No growth	0	Liver shows 1 nodule as in Pigeon 73
81	1 week	No growth	0	Moderate increase of round cells in liver, grouped about which are a few adult polymorphonuclear leucocytes and an occasional myelocyte. Many small basophilic cells seen about vessels and in interstitial tissue of kidney. Moderate hyperplasia of bone marrow
82	1 week	No growth	0	Periportal round cells of liver moderately increased. Also a few polymorphonuclear leucocytes and an occasional myelocyte seen in these areas. No increase of round cells in kidney but 1 small patch of myelocytes seen
84	1 week	No growth	0	No lesions
85	1 week	No growth	0	No lesions
88	1 week	No growth	0	No lesions

As seen in the table, cultures of the liver made upon blood agar plates were universally sterile, but the sera of two of the pigeons which had been kept for a long period of time in close proximity to birds infected with *B. aertrycke* were found to agglutinate *B. aertrycke* in dilutions of 1:64 and 1:32 respectively. No agglutinins were present in the bloods of any of the other birds. Attention is called to the frequency with which pathological changes are found in the liver and kidneys of apparently normal pigeons, also to the fact that occasional myelocytes are present in the tissues of such birds. No noteworthy changes of any kind were found in the bone marrow, lungs, spleen, testes, ovaries and intestine.

Studies upon Pigeons Surviving Experimental Infection with B. Aertrycke

Bacteriological, serological and anatomical studies have been made upon eight pigeons which survived experimental infection with *B. aertrycke* for periods of time varying from five to nine months. All of these pigeons were well nourished and showed no alterations in the peripheral blood or other manifestations of disease when killed for study. Each of these birds had, however, become clinically ill, had lost weight and shown marked increase of the myeloid elements of the blood for a period shortly after oral administration of *B. aertrycke.*

Bacteriological cultures made routinely from the liver on blood agar plates were universally sterile, and cultures of the intestinal contents and mucosa failed to show pathogenic organisms of any kind.

The results of the serological studies are shown in Table IV.

The changes found in the tissues of these pigeons, though of considerable extent, cannot be fully understood until the histogenesis of the lesions produced by *B. aertrycke* has been studied further. Briefly stated, they consisted almost entirely of the healing of the destructive lesions caused by the bacteria. Pigeon 15, killed nine months after infection, showed only one small, encapsulated, caseous area in the lung, the remains of a small patch of pneumonia, no changes of any kind being present in any of the other tissues. The other seven pigeons, killed five months after infection, showed many large phagocytic cells in the liver and spleen loaded with iron

pigment, numerous healing necroses in the liver, and many circumscribed caseous areas in the lungs, each surrounded by a wall of giant cells and connective tissue. These latter lesions, obviously the result of the characteristic lobular pneumonia produced by *B. aertrycke*, varied from microscopic size to as much as 7 mm. in diameter. The smaller ones were identical with the larger healing necroses found in

TABLE IV

Agglutination Titer of Blood Serum to B. Aertrycke

No.	Before infection	After infection		
		1 month	5 months	9 months
15	0	..	1:1280	0
16	0	0	1:160	..
20	0	1:2560	0	..
22	0	1:80	0	..
25	0	1:1260	0	..
36	0	0	0	..
38	0	1:320	0	..
42	0	1:320	1:80	..

the liver and also bore close resemblance to the nodular lesions frequently found in the liver of apparently normal pigeons. There was almost no trace left of the myeloid hyperplasia which may have existed during the phase of acute disease in this series of pigeons. In three birds, quite numerous small groups of adult polymorphonuclear leucocytes were seen about the blood vessels in the liver. The bone marrow had returned to its normal appearance in six of the pigeons, but in the two remaining birds gelatinous degeneration of the marrow of the radius was present.

DISCUSSION

A number of previous workers have already called attention to unusually high leucocytoses varying from 200,000 to 600,000 cells per cmm. which characteristically occur in birds during the course of certain bacterial infections. Among these may be mentioned Smith and Moore,[6] who described a disease of fowls caused by an organism to which they gave the name *B. sanguinarium*, Burckhardt,[7] and Hirschfeld and Jacoby,[8, 9] who studied fowls infected with

avian tuberculosis. So great was the number of myeloid cells in the blood that Smith and Moore considered the disease which they studied to be a form of myeloid leukemia, while Burckhardt expressed the view that myeloid leukemia in fowls was a manifestation of tuberculosis. Hirschfeld and Jacoby oppose Burckhardt's opinion. We can find no blood studies upon pigeons infected with tuberculosis but have ourselves observed a pigeon dying of naturally acquired tuberculous infection whose blood contained 185,000 leucocytes per cmm., of which 92 per cent were mature polymorphonuclears. Cultures of the blood and organs of this bird on blood agar plates and broth were sterile. Though the bone marrow showed marked myeloid hyperplasia, the cells present were mostly adult leucocytes or myelocytes well advanced toward maturity. No myelocytes were seen in the blood and there was no infiltration of other organs with myeloid cells. The mouth, pharynx and bones of the paranasal sinuses showed large caseous lesions, while the liver and spleen contained countless small tubercles. In all of these lesions masses of acid-fast bacilli were present.

None of the observers who have studied these hyperleucocytoses of birds caused by bacteria has reported the presence of an appreciable number of immature white cells in the peripheral blood or the occurrence of infiltrations of such cells in the viscera. We therefore agree with Opie,[10] who recently expressed the view that those investigators reporting the experimental production of diseases closely related to leukemia by means of bacterial infection have failed to justify their claim.

In the case of both natural and experimental infection of pigeons with *B. aertrycke*, however, the situation seems to us to be somewhat different from that of other bacterial infections of birds hitherto reported, though we are by no means prepared to state that this disease is in any way fundamentally related to myeloid leukemia of birds, mammals or human beings. At the present stage of our studies of *B. aertrycke* infection in pigeons we can call attention only to the common occurrence of large numbers of immature myeloid cells in the peripheral blood and the constant appearance of extensive myeloid foci in the liver and kidneys of birds infected with this organism. These changes, to our knowledge, have not been observed to occur in other bacterial infections. We wish to emphasize the fact that, in addition to the hyperplasia and heterotopia of the myeloid tissue

here described, in which no bacteria were found, other widely spread lesions of a totally different character, consisting of accumulations of mononuclear phagocytic cells, containing *B. aertrycke* in abundance, occurred with regularity.

Inasmuch as we have not yet had the opportunity of studying fowl leukemia we can make no definite statement concerning the relationship of this disease to *B. aertrycke* infection in pigeons, though one would be inclined to consider the two conditions as separate entities. Most of our knowledge of fowl leukemia is derived from the studies of Ellermann and Bang,[11] Ellermann,[12, 13, 14, 15] Hirschfeld and Jacoby,[8, 9] and Schmeisser,[16, 17] who, in general, agree that the disease is caused by a filterable microörganism. Ellerman and Bang, who first advanced this theory, did so upon the ground that they were able to transmit the disease to a certain percentage of normal fowls by the injection of bacteria-free filtrates prepared from the organs of a fowl dying of the disease spontaneously acquired. These authors, however, do not appear to have searched very thoroughly for bacteria. No mention is made of cultures made directly from the organs of the birds spontaneously or experimentally infected, nor were the tissues of these birds examined microscopically for bacteria. The only proof offered for the sterility of the filtrate with which the disease was experimentally produced is the statement that it was clear and gave rise to no growth when cultured in several types of media. The amount of filtrate cultured was not mentioned, but it was stated that cultures of the emulsion of tissues from which the infectious filtrate was made gave a rich growth of bacteria. No further study was made of these organisms. Though it is possible that the filtrate with which Ellermann and Bang claimed to have produced leukemia in fowls was actually free of bacteria, one does not feel convinced that such was the case from the account of their experiments. Hirschfeld and Jacoby,[9] who attempted to repeat the experiments of Ellermann and Bang,[11] began their studies with a leukemic fowl obtained directly from the latter workers and were apparently successful in transmitting the disease to other fowls by injections of whole organ emulsion prepared from the diseased bird. However, their stock of fowls proved to be heavily infected with spontaneously acquired tuberculosis. The results of their experiments are not altogether clear, though they studied great numbers of birds in their attempt to separate the effects of these two diseases. It is

worthy of note that Hirschfeld and Jacoby did not succeed in producing leukemia in any of their fowls by the injection of bacteria-free filtrates made from the organs of leukemic birds. Schmeisser,[16, 17] has studied a disease of fowls considered to be myeloid leukemia, which he found to be transmissible to normal birds by injection of organ emulsions made from both spontaneously and experimentally infected birds. He has written a good description of the changes in the blood and other organs but in neither of his papers has he mentioned any bacteriological studies whatever.

Just as this paper was completed the publications of Furth and Stubbs,[33, 34] appeared, confirming once more the filterable nature of the etiological agent in fowl leukemia. While the similarity is striking in certain phases of the hemopoietic response, it would seem that entirely different causative agents were at work in fowl leukemia and *B. aertrycke* infection in pigeons. Though all of these investigators who have studied fowl leukemia have described lesions apparently identical with the changes in the myeloid tissue occurring in pigeons infected with *B. aertrycke*, none has reported the presence of the widely spread collections of monocytic cells frequently grouped about bacteria which we have regularly observed. It seems unlikely that such lesions would be overlooked. Furthermore, whereas the incubation period of fowl leukemia is stated to be approximately two months, our pigeons infected with large doses of *B. aertrycke* became ill within two days, and small doses of these bacteria produced no symptoms of disease at all. In relation to this latter point may be mentioned the work of Winternitz and Schmeisser,[18] who studied a series of fowls experimentally infected with *B. sanguinarium*. As previously found by Smith and Moore, their birds developed high leucocytoses but showed no extramedullary accumulations of myelocytes. A single bird, however, developed changes in the blood characteristic of myeloid leukemia several weeks after injection of the bacteria and was found to show extensive infiltration of the liver and kidneys with myelocytes. No bacteria of any kind were recovered from this bird and these investigators were inclined to conclude that the case was one of true fowl leukemia. They suggested the possibility that leukemia may be produced in the fowl by graded doses of *B. sanguinarium*, but did not pursue the matter further.

Aside from its possible relationship to fowl leukemia, the question

of *B. aertrycke* infection in pigeons is, in itself, a matter of considerable interest. Our observations, though inadequate as absolute proof, would point strongly to the assumption that pigeons frequently harbor minimal numbers of this organism which, under ordinary conditions, cause them no harm. However, under adverse conditions, such as severe malnutrition, it would seem that these bacteria might multiply and frequently become sufficiently pathogenic to produce disease.

That *B. aertrycke* infection is a natural disease of pigeons is shown by observations recently made upon a bird noted to be seriously ill when brought into the laboratory. Immediate examination of the blood showed 2,900,000 erythrocytes and 108,000 leucocytes per cmm., eosinophilic leucocytes 64 per cent, myelocytes 2 per cent, lymphocytes 20 per cent, and monocytes 14 per cent. A culture of the blood made at the same time was positive for *B. aertrycke*, which was identical in all of its characteristics with the strains isolated from spontaneous cases of *B. aertrycke* infection developing in pigeons in the laboratory in New York. On the following day a second blood culture was also positive for *B. aertrycke*. This pigeon was isolated from the other birds and observed for eight days, during which time it remained critically ill, continued to lose weight, and the granulocytes of the blood increased to 83 per cent. After death on the eighth day, all of the lesions characteristic of *B. aertrycke* infection were found in advanced degree and the organism was recovered from the liver, kidney, bone marrow and blood. Intraperitoneal injection of saline emulsion made from the liver, as well as pure cultures of the bacteria, produced both symptons and lesions in normal pigeons identical with those occurring after experimental infection with other strains of *B. aertrycke*.

We have not yet studied the effect of oral administration of *B. aertrycke* upon animals other than pigeons, but have found these organisms to be highly pathogenic for chickens, mice, guinea pigs and rabbits when injected in fairly large doses. The animals died of septicemia within a few days but did not show myeloid alterations in their organs such as those seen in pigeons infected by the oral route.

Though *B. aertrycke* is generally mentioned in the literature in association with food poisoning in man, it also has been observed to cause disease in animals. The system of nomenclature for the group

of bacteria related to *B. aertrycke* is so inaccurate, however, that relatively few of the investigators who have reported such observations appear to have been dealing with the same organism. Otte [19] has reviewed the many studies upon natural and experimental infection of birds with bacteria of this type, and Beck and Meyer [20] have studied a disease of pigeons, widespread in Germany, which was caused by a bacillus considered to be *B. aertrycke*. No blood studies were made by any of these observers. Infiltrations with mononuclear cells, similar to those we have observed in pigeons, were of common occurrence but no changes in the myeloid cells were reported.

Beaudette and Edwards [21] have reported an epidemic in canaries caused by this organism, Beaudette [22] an epidemic in young pigeons, and Doyle [23] has described an epidemic among young chicks. Meyer and Matsumura [24] state that a considerable percentage of wild rats are carriers of *B. aertrycke*. Petrie and O'Brien [25] described an epidemic in guinea pigs which they thought was due to a filterable microörganism, but regularly cultured *B. aertrycke* from the dead animals. This organism was highly pathogenic for normal guinea pigs when injected subcutaneously, but of low virulence when administered by mouth. Topley and Ayrton,[26] working with white mice, found the greatest variation in the behavior of different strains of *B. aertrycke*. Some strains were excreted regularly from the intestine after oral administration, while others appeared only transiently, in some instances fatal infection taking place without the organisms ever appearing in the feces. These workers also showed that *B. aertrycke* frequently remained in the tissues of mice without causing disease. Topley,[27] at a later date, demonstrated *B. aertrycke* of unaltered virulence in the spleens of mice actively immunized to lethal doses of this organism. Edington [28] has recently made a very thorough study of another guinea pig epidemic caused by *B. aertrycke* in which this organism was recovered regularly from the blood, intestine, gall-bladder, liver, spleen and urine. Blood studies showed a leucopenia varying from 2,000 to 4,000 cells per cmm. with no striking change in the differential count. The lesions found were empyema of the gall-bladder, necrosis in the spleen and liver, hyperemia of the intestine and occasional small ulcerations in the lymphoid follicles of the mucosa, accumulations of phagocytes with necroses in the mesenteric lymph nodes, catarrhal inflammation of urinary

bladder, purulent endometritis, infrequent pneumonia and cloudy swelling of kidneys. No mention was made of the bone marrow.

Though the literature contains several references,[29, 30, 31] other than instances of food poisoning, to human cases of fatal infection with *B. aertrycke*, none of them has resembled the disease caused by this organism which we have studied in pigeons. From the accounts of such cases it seems that *B. aertrycke* was recovered only from the blood, and was never proved to be the cause of any of the lesions present.

Summary and Conclusions

The apparently spontaneous development of a fatal disease in undernourished pigeons is reported which is characterized by anemia, marked myeloid hyperplasia of the bone marrow, striking increase of the myeloid elements of the blood, and extensive infiltration of the liver and kidneys with myeloid tissue. In addition to these myeloid changes, large, nodular, often necrotic masses of mononuclear phagocytic cells are frequently found scattered throughout the liver, spleen, kidneys and bone marrow.

A small, Gram-negative bacillus, regularly recovered in pure culture from the blood, liver, kidney, spleen, and bone marrow of these cases, has been identified as *B. aertrycke*. In sections, the bacteria are found to be present in the foci of mononuclear cells, but do not occur within the collections of myelocytes.

Disease has been produced experimentally in normal pigeons by the intraperitoneal injection of liver emulsion made from naturally infected birds, intraperitoneal injection of *B. aertrycke* derived from the same source, and also by oral administration of single large doses of broth cultures of this organism.

Bacteria-free filtrates of broth cultures of *B. aertrycke* have had no demonstrable effect upon normal pigeons when injected or administered orally in single large doses.

Attention is called to the frequency with which pathological changes occur in the tissues of apparently normal birds.

Note: The authors wish to extend their warmest thanks to Dr. L. T. Webster, of the Rockefeller Institute, and Dr. T. J. Kurotchkin, of the Department of Bacteriology of the Peiping Union Medical College, for their kind assistance with the bacteriological and serological studies reported in these experiments.

REFERENCES

1. Sabin, F. R. *Bull. Johns Hopkins Hosp.*, 1923, **34**, 277.
2. Forkner, C. E. *J. Exper. Med.*, 1929, **50**, 121.
3. Klieneberger, C., and Carl, W. Die Blut-Morphologie der Laboratoriums-Tiere. Barth, Leipzig, 1912.
4. Arloing and Dufourt. *Soc. sc. vét., J. de Med. vét. et de zootech.*, Lyon, May, 1922. (Quoted by De Eds.)
5. De Eds, F. *J. Lab. & Clin. Med.*, 1926, **12**, 437.
6. Smith, T., and Moore, V. A. *Annual Report, U. S. Bureau Animal Industry*, 1895, Bull. 8.
7. Burckhardt, J. L. *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, 1912, **14**, 544.
8. Hirschfeld, H., and Jacoby, M. *Ztschr. f. klin. Med.*, 1912, **75**, 501.
9. Hirschfeld, H., and Jacoby, M. *Ztschr. f. klin, Med.*, 1910, **69**, 107.
10. Opie, E. L. *Medicine*, 1928, **7**, 31.
11. Ellermann, V., and Bang, O. *Centralbl. f. Bakteriol.*, 1908, **46**, 4 and 595: *Ztschr. f. Hyg. u. Infektionskrankh.*, 1909, **63**, 231.
12. Ellermann, V. *Verhandl. d. deutsch. path. Gessellsch.*, 1908, **12**, 224.
13. Ellermann, V. *Ztschr. f. klin. Med.*, 1913, **79**, 43.
14. Ellermann, V. *J. Exper. Med.*, 1921, **33**, 539.
15. Ellermann, V. The Leucoses of the Fowl and Leucemia Problems. Gyldendal, London, 1921.
16. Schmeisser, H. C. *J. Exper. Med.*, 1915, **22**, 820.
17. Schmeisser, H. C. *Johns Hopkins Hosp. Rep.*, 1916, **17**, 551.
18. Winternitz, M. C., and Schmeisser, H. C. *Johns Hopkins Hosp. Rep.*, 1919, **18**, 25.
19. Otte, W. Die Krankheiten des Geflügels mit besonderer Berücksichtigung der Anatomie und der Hygiene. Schoetz, Berlin, 1928.
20. Beck, A., and Meyer, E. *Ztschr. f. Infectionskr. d. Haustiere*, 1926, **30**, 15.
21. Beaudette, F. R., and Edwards, P. R. *J. Bact.*, 1926, **12**, 51.
22. Beaudette, F. R. *J. Am. Vet. M. A.*, 1925-26, **68**, 644.
23. Doyle, T. M. *J. Comp. Path. & Therap.*, 1927, **40**, 71.
24. Meyer, K. F., and Matsumura, K. *J. Infect. Dis.*, 1927, **41**, 395.
25. Petrie, G. F., and O'Brien, R. A. *J. Hyg.*, 1910, **10**, 287.
26. Topley, W. W. C., and Ayrton, J. *J. Hyg.*, 1924, **22**, 234.
27. Topley, W. W. C. *Lancet*, 1929, **1**, 1337.
28. Edington, J. W. *J. Comp. Path. & Therap.*, 1929, **42**, 258.
29. Shaw, F. W. *J. Lab. & Clin. Med.*, 1926, **12**, 141.
30. Bullowa, J. G. M. *M. Clin. N. Amer.*, 1928, **12**, 691.
31. Hu, C. K. *Nat. M. J. China*, 1929, **15**, 149.
32. Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A. *J. Exper. Med.*, 1924, **40**, 845.
33. Furth, J. *J. Exper. Med.*, 1931, **53**, 243: *Proc. Soc. Exper. Biol. & Med.*, 1931, **28**, 449.
34. Stubbs, E. L., and Furth, J. *J. Exper. Med.*, 1931, **53**, 269.

DESCRIPTION OF PLATES

PLATE 67

Cells stained supravitally with neutral red and Janus green from the peripheral circulation of pigeons in this experimental series.

FIG. 1. Young adult eosinophilic leucocyte showing mitochondria and a mixture of spheres and rods making up the specific granules. These are the first changes to appear in the circulating granulocytes of the pigeon under stress. Pigeon 70, April 16, 1929, size 9 × 12 microns.

FIG. 2. Young adult eosinophilic leucocyte with mitochondria. Pigeon 65, peripheral blood, April 3, 1929, size 9 × 9½ microns.

FIG. 3. Atypical micro-eosinophil with swollen rods not infrequently found in the circulation as an indication of bone marrow stimulation. Pigeon 65, April 3, 1929, size 8 × 9 microns.

FIG. 4. Adult eosinophilic leucocyte showing the incomplete transition of spherical granules to rods and indicating a premature delivery of this cell to the circulation. Pigeon 65, April 3, 1929, size 10 × 15 microns.

FIG. 5. Eosinophilic myelocyte "B" with round nucleus, mitochondria and a moderate number of specific, spherical granules. Pigeon 65, April 3, 1919, size 10 × 10 microns. See erythrocyte for relative size.

FIG. 6. Eosinophilic myelocyte "A" with many mitochondria and few specific granules. Size 9 × 10 microns. Note the different stages of maturation found within the eosinophilic group on the same day from Pigeon 65, April 3, 1929.

FIG. 7. Eosinophilic myelocyte "C," showing mitochondria and spherical granules arranged about the centrosphere, a not infrequent arrangement in the myelocytic stage. The slightly indented achromatic nucleus contains one nucleolus. Pigeon 50, March 22, 1929, size 10½ × 12 microns.

FIG. 8. Myelocyte "B" contains approximately one-half the final concentration of specific granules found in the fully mature eosinophil and many mitochondria. The area about the centrosome is clear of granules. The nucleus shows very little chromatin structure at this stage of development. Pigeon 50, March 22, 1929, size 15 × 15 microns.

FIG. 9. This myelocyte "A" has relatively few specific granules, several vacuoles, and many mitochondria. The nucleus contains two nucleoli and very little chromatin. Size 12 × 13½ microns. Cells 7, 8 and 9 show the various stages of myelocytic maturation found on the same day in the blood of Pigeon 50, March 22, 1929.

FIG. 10. Myelocyte "B." Pigeon 65, April 4, 1929, size 9 × 12 microns.

FIG. 11. Myelocyte "A." Pigeon 65, April 4, 1929, size 10 × 14 microns.

FIG. 12. Monocyte from the peripheral blood of Pigeon 50, March 21, 1929. Note the slightly different shade of neutral red reaction in the vacuoles of this cell as contrasted with the granules in the myelocytes of Figs. 7, 8 and 9 from the same bird. See accompanying erythrocyte for relative size.

FIG. 13. Monocyte from Pigeon 65, April 4, 1929. Contrast this cell with the myelocytes of Figs. 10 and 11 taken from the same bird on the same date. Size 9 × 10½ microns.

FIG. 14. Small lymphocyte with many mitochondria and a few scattered neutral red vacuoles. Pigeon 67, April 4, 1929, size 9 × 9 microns. See accompanying erythrocyte for relative size.

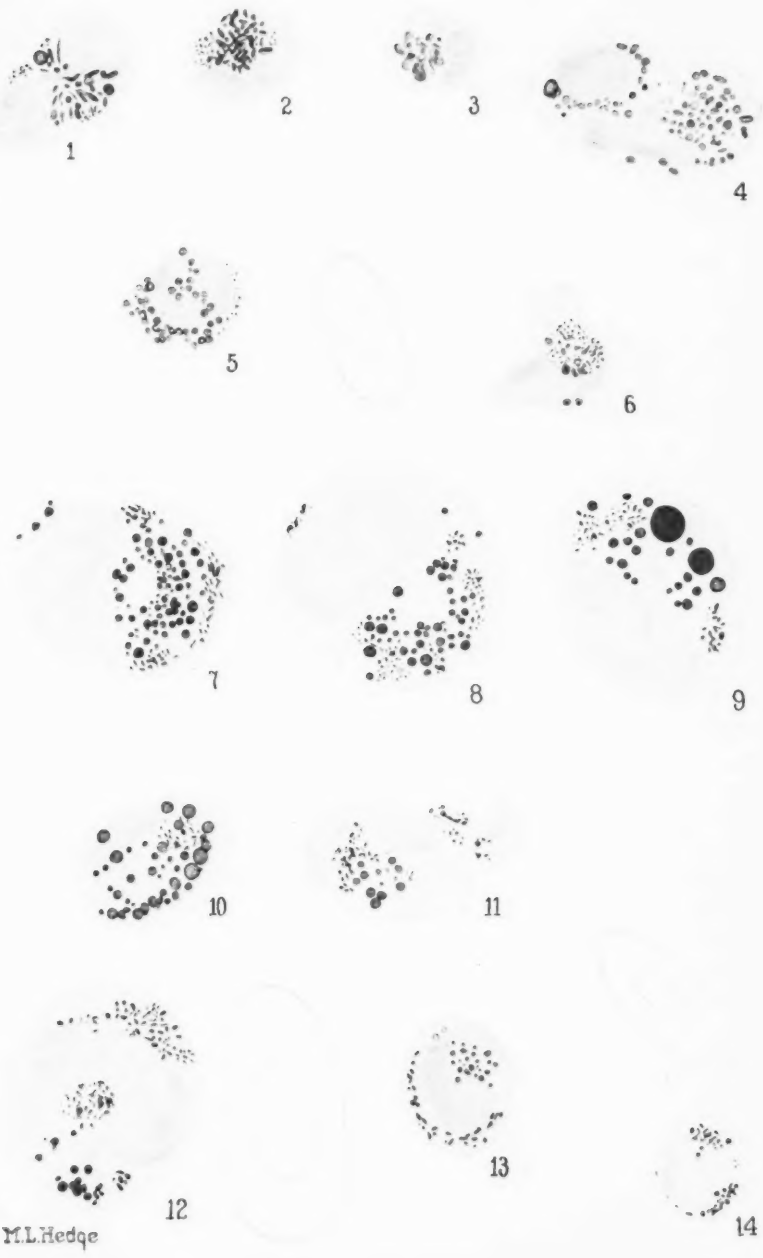

1
2
3
4
5
6
7
8
9
10
11
12
13
14
M.L.Hedge

PLATE 68

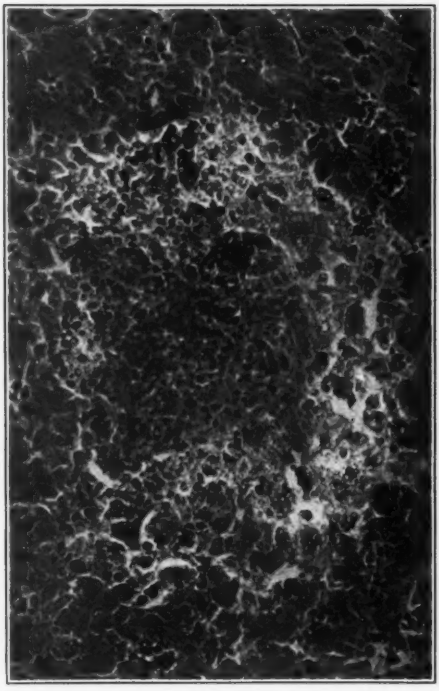
FIG. 15. Liver of Pigeon R714, naturally infected with *B. aertrycke*, showing unusually large collection of monocytic cells, in which there is a necrotic area containing Gram-negative bacilli. Hematoxylin-eosin stain. × 62.

FIG. 16. Smaller collection of monocytic cells in liver of Pigeon 24, experimentally infected with *B. aertrycke*. There are bacteria and a minute area of necrosis in the center about which is heavy infiltration with adult polymorphonuclear leucocytes. Hematoxylin-eosin stain. × 300.

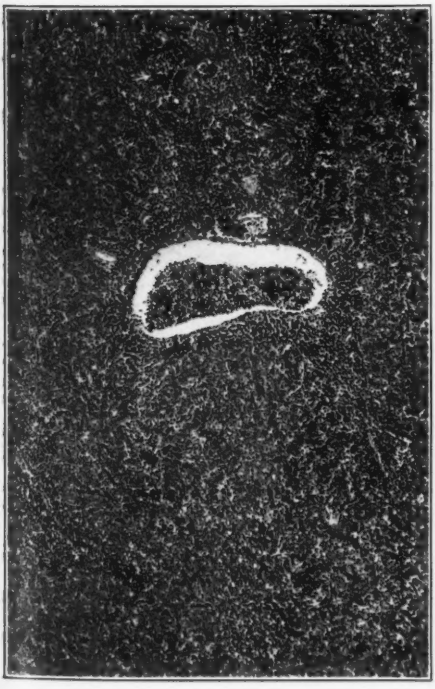
FIG. 17. Low power picture of liver of Pigeon 14, experimentally infected with *B. aertrycke*, showing general distribution of infiltrations of myeloid cells. × 62.

FIG. 18. Small branch of portal vein with surrounding zone of myelocytes. Experimental infection *B. aertrycke*, Pigeon 14. Hematoxylin-eosin stain. × 300.

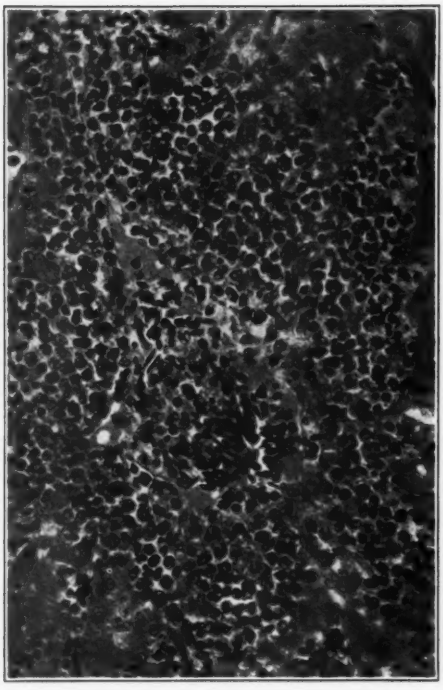
15



16



17



18

Cash and Doan

Infection of Pigeons with B. Aertrycke

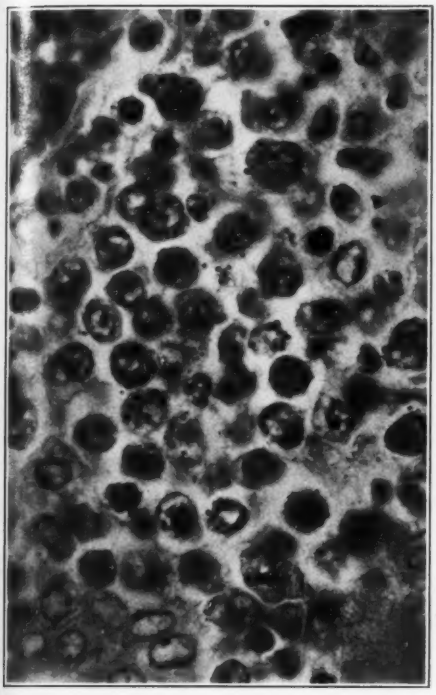
PLATE 69

FIG 19. Myelocytes surrounding portal vein. A small portion of the vessel filled with erythrocytes may be seen in the upper left-hand corner. Natural infection with *B. aertrycke*, Pigeon 14. Hematoxylin-eosin stain. × 1300.

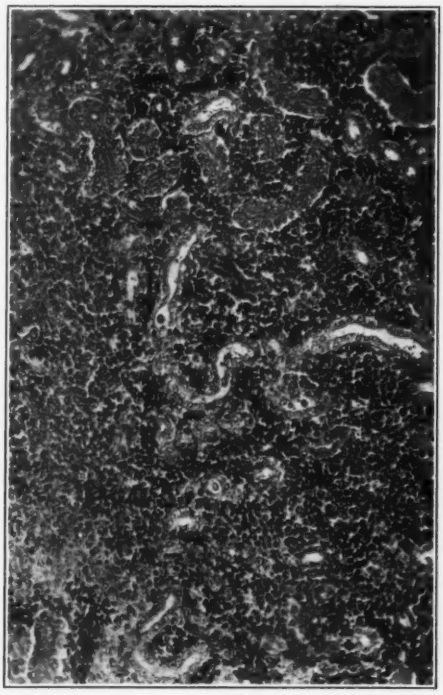
FIG. 20. Infiltration of kidney with myelocytes. Experimental infection with *B. aertrycke*, Pigeon 14. Hematoxylin-eosin stain. × 158.

FIG. 21. Bone marrow from central portion of shaft of radius of Pigeon 30 dying of experimental *B. aertrycke* infection. The partially collapsed capillaries are marked out by the characteristic nuclei of erythrocytes. All of the remaining cells are myelocytes of varying degrees of maturity. The more mature forms appear darker than the others due to their greater number of granules. The degree of myeloid hyperplasia may be appreciated by bearing in mind the fact that the capillary network of this portion of the marrow of normal pigeons contains only fat among its meshes. Hematoxylin-eosin stain. × 300.

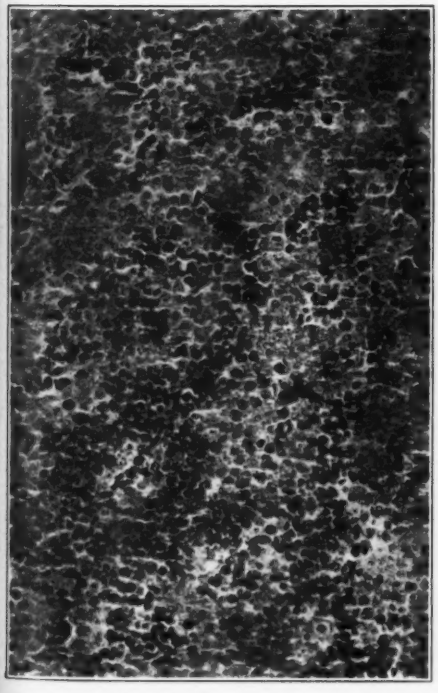
FIG. 22. Oil-immersion picture from same marrow as Fig. 7, illustrating the pale staining, myeloid forms with only a few granules in their cytoplasm. A few mature myelocytes with their full quota of granules are also seen. Hematoxylin-eosin stain. × 1300.



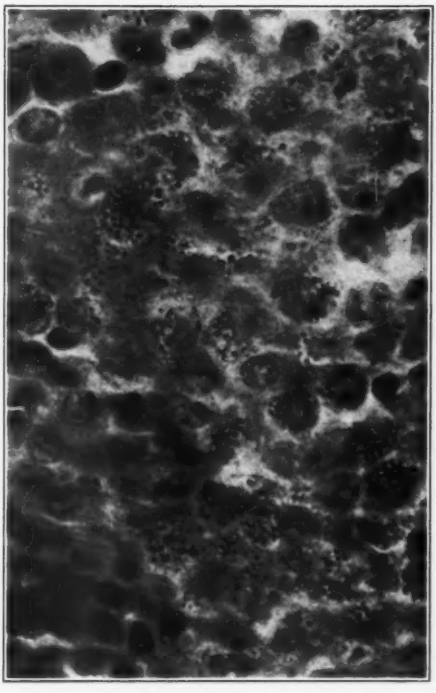
19



20



21



22

Cash and Doan

Infection of Pigeons with B. Aertrycke

A SYSTEMATIC STUDY OF THE DEGENERATION OF ARTICULAR CARTILAGE IN BOVINE JOINTS *

GRANVILLE A. BENNETT, M.D., AND WALTER BAUER, M.D.
(From the Department of Pathology, Harvard Medical School, and the Medical Clinic of the Massachusetts General Hospital, Boston, Massachusetts)

In a previous study concerning the cytology and nitrogen content of normal synovial fluid of cattle,[1] constant differences were noted in the fluid removed from the carpometacarpal and astragalotibial joints. Synovial fluid obtained from the carpometacarpal joints was more viscid, contained more nucleated cells per cubic millimeter and showed a higher total protein content than did synovial fluid obtained from the astragalotibial joints of the same animal. At that time, occasional macroscopic and microscopic examinations of these joints revealed constant areas of degeneration in the medial articular cartilages of the carpometacarpal articulations. The astragalotibial joints which were examined [1] did not reveal similar lesions. A brief comment concerning these areas of degeneration in cartilage was made. The constant differences in the synovial fluid obtained from these carpometacarpal joints as compared to the synovial fluid aspirated from the astragalotibial articulations was explained by their presence.

The present investigation was undertaken with the purpose of studying these degenerative changes in the articular cartilage from their beginning through all the stages of development and if possible of assigning the causes for their occurrence. It was also hoped that a detailed study of the initial lesions might enable us to understand better the earliest pathological changes which occur in diseases of the articular cartilage in man.

A study was made of the carpometacarpal joints in a series of embryos in order to determine whether or not constant differences in development or peculiarities in vascular supply played any part in the production of these lesions.

* This is publication No. 3 of the Robert W. Lovett Memorial for the study of crippling diseases, Harvard Medical School, Boston, Massachusetts.
Received for publication May 8, 1931.

Material and Methods

In all, two hundred and seventeen carpometacarpal joints of bovine embryos, calves and cattle were examined. The joints of eight embryos ranging from 5.2 cm. to 70 cm. in length were subjected to macroscopic and microscopic examination. Thirty joint specimens obtained from slaughtered calves were examined grossly. Histological studies of selected ones were made. The joints of one hundred steers and heifers between 1 and 5 years of age and of fifty older milch cows were examined macroscopically. Finally, thirty-seven specimens which showed the minimal to maximal sized lesions in the two types of animals, young steers and heifers (beef cattle) and the older milch cows, were selected. All of this latter group of specimens were used for macroscopic and microscopic study. From them, the gross and microscopic illustrations were made.

The specimens were all fixed in 10 per cent formaldehyde solution. Large or complete transverse or anteroposterior blocks of cartilage and subchondral bone were taken. These blocks were decalcified in a 5 per cent nitric acid solution and embedded in celloidin. Sections were stained with hematoxylin and eosin. A few of the embryo specimens were embedded in paraffin, as were the synovial membrane specimens. Serial sections were made from a few of the celloidin blocks, employing a modification of the technique described for use in frozen sections.[2]

Development of Articular Cartilage in the Carpometacarpal Joint

Macroscopic Description: The carpometacarpal joints of a 5.2 cm. embryo were too small to warrant gross description. In a 15 cm. embryo, the metacarpal articular cartilage measured 3.5 mm. in width by 2 mm. in depth. In the larger embryos, the articular cartilage had increased sufficiently in size, so that in the oldest (70 cm.) embryo, it measured 30 mm. in width by 3 to 3.5 mm. in thickness. Small blood vessels just visible to the unaided eye were seen in all the larger specimens of articular cartilage.

The macroscopic examination of the carpometacarpal joints of thirty-five calves which were approximately 6 to 12 weeks of age failed to reveal any evidence of articular cartilage degeneration. The surface of the cartilage was smooth and glistening (Fig. 1). Occa-

sional small blood vessels could be seen in the depth of the cartilage by superficial inspection. After shaving off the surface cartilage, a rich blood vessel and capillary network was seen readily with the aid of a hand lens. Vertical transverse cross-sections showed the articular cartilage to average approximately 1 mm. in thickness. The metacarpal articular cartilage in animals older than those already described was reduced to a fraction of a millimeter in thickness. It should be emphasized that in the metacarpal bones of the cow there is no epiphysis. Thus, the thick articular cartilage of the embryo gradually becomes transformed into a thin adult articular cartilage.

Microscopic Description: A longitudinal section through the fore leg of the smallest (5.2 cm.) embryo showed the bulk of tissue to consist of cell-poor embryonic connective tissue. In the area where the carpometacarpal articulation later develops, there had been an accumulation of mesenchymal cells. This compact group of cells had differentiated, in its central portion, into avascular embryonal cartilage, while the peripheral zone of cells appeared to consist wholly of fibroblasts. No cleavage into a joint space had occurred in the region of the carpometacarpal joint, although a well formed articular cavity had developed at the radiohumeral joint. With the exception of the humerus, where some bony matrix had been laid down, the bone anlage consisted wholly of avascular embryonal cartilage. A few blood vessels, however, were noted in the surrounding cellular connective tissue (periosteum).

Longitudinal sections through the carpometacarpal joints of embryos 15 to 59 cm. in length revealed well developed bones, articular cartilages and joint cavities. In these specimens the cartilage had differentiated into three zones: (1) a superficial or perichondral, (2) a middle or vascular, and (3) a deep or proliferating zone (see Fig. 2). These three zones merged one into another without sharp lines of distinction. Numerous mitotic figures were found in all three zones. The surface zone of cartilage consisted of three to five layers of cells, the most superficial layer of which had the morphology of fibroblasts. The middle zone was composed of irregularly shaped and placed embryonal cartilage cells. Several medium sized blood vessels were present in this zone. These blood vessels, which usually consisted of artery and accompanying vein, could be traced in all sections except two to the larger vessels in the perichondrium and periosteum at the margins of the joints and the line of fusion between the meta-

carpal bones. The deepest zone of cartilage consisted of columns of flattened cells; in the upper two-thirds the cells were rapidly dividing, whereas in the lower one-third the cells were undergoing degeneration, prior to bone formation.

Embryos of 59 and 70 cm. length showed articular cartilages which had markedly increased in width. Evidences of rapid growth were still present in the form of fairly numerous mitotic figures and incomplete ossification of the subchondral bone trabeculae. Blood vessels of large size were seen entering from the margins of the cartilage into its middle zone where they immediately branched into many smaller divisions. One noted in the study of these serial sections that many of the blood vessels just above the line of ossification were obliterated. No blood vessels were seen to enter from the subchondral bone.

Sections obtained from growing calves showed a thinner cartilage which was mature from the standpoint of both cells and matrix (see Fig. 3). Fewer blood vessels were present. The three merging layers of cells were less distinct. The cartilage cells were nearly always in pairs or groups of cells and were arranged in an orderly manner. No mitotic figures were seen. A more abundant matrix was present and the subchondral bone was more completely calcified. The line of ossification in all preceding specimens was even and (in brief) consisted of buds of capillaries growing into the degenerating columns of cartilage cells. Oval, elongated and polyhedral cells (osteoblasts) accompanied these blood vessels and a pink-staining homogeneous osteoid matrix was laid down about them. This matrix was built up into regular, evenly spaced bone trabeculae. In the specimens obtained from young calves, the subchondral bone had encroached sufficiently upon the vascular zone of cartilage, so that occasional blood vessels were being surrounded by bone above the line of ossification (Fig. 3). This overtaking of the vascular zone of articular cartilage by subchondral bone suggests the process by which adult cartilage becomes avascular. In all the embryo specimens, the transition of cartilage into bone was by direct replacement from below. In calves, a calcified layer of cartilage had begun to form (Fig. 3). In older animals the deepest layer of cartilage was calcified, as indicated by its staining reaction (Fig. 4).

Degenerative Lesions of Articular Cartilage

Macroscopic Examination: In order that the stages of development of these degenerative lesions of cartilage might be more briefly and clearly described, the specimens have been grouped into four classes for both gross and microscopic study: (1) early lesions as illustrated in gross photographs, Figs. 5, 6, 7 and 8; (2) medium sized lesions as in Fig. 9; (3) large lesions of older beef cattle as in Figs. 10 and 11, and finally, (4) defects seen in old milch cows as illustrated in Figs. 12, 13 and 14.

Gross examination of the carpometacarpal articulations of six steers and heifers (beef cattle), which because of the absence of any permanent teeth were assumed to be under 2 years of age, revealed the earliest lesions. These changes are illustrated in Figs. 5, 6, 7 and 8. They consisted of slightly depressed, roughened areas of cartilage, or small areas in which cartilage was completely absent (Fig. 8). These small erosions always occurred in the concave surface of the medial articular cartilage.

Larger and deeper areas of degeneration in articular cartilage were found in the other carpometacarpal joints collected. Fig. 9 illustrates a degenerative lesion of average size and depth for young beef cattle. The area of degeneration in this joint measured 10 by 11 mm. It was sharply outlined by an irregular margin of overhanging cartilage. The base of the defect extended into subchondral bone 1.5 mm. in the deepest portion.

Figs. 10 and 11 illustrate the largest degenerative lesions observed in beef cattle. These lesions measured 20 by 17 by 2.5 mm. and 25 by 7 by 5 mm. respectively. In both instances they had extended deeply into the subchondral bone. The similarity of the size, shape and location of the lesions in the carpal cartilage as compared to the defects in the metacarpal cartilage is well illustrated in Fig. 11. It should be emphasized that these degenerative changes always occurred on the medial half of the joint cartilage and were present in both the carpal and metacarpal cartilages. Usually one defect was the mirror image of the opposing degenerative area and striking similarities between right and left side were noted (see Fig. 10).

In old milch cows, the degenerative lesions, while located in the same areas, presented slightly different appearances. The margins were sharper and less irregular in outline. The surrounding articular

cartilage was a pale yellow in color. Various sized lesions in old milch cows are illustrated in Figs. 12, 13 and 14. The bases of several of the larger defects appeared on macroscopic examination to be covered by organizing fibrin. Histological study, however, failed, with one exception, to show any fibrinous exudate.

In occasional joints, at the site of the future degenerative lesions, minute, hard yellowish elevations were found. These nodules did not usually project more than 1 mm. above the cartilage surface and were seldom more than 2 mm. in diameter.

Macroscopically there was little evidence of synovial membrane pathology. No villous fringes were seen and for the most part the synovial membrane was smooth and glistening.

Microscopic Examination: A systematic study of sections from twenty-two joints was made. These sections showed all stages of development of the articular cartilage lesions.

The normal portions of the adult articular cartilage showed a thin layer of uniform hyaline cartilage, which was 0.7 to 0.9 mm. in thickness. The articular surface was smooth. At the surface the paired cartilage cells, which were usually two to four layers deep, were so arranged that their long axis was transverse to the vertical axis of the bone. Beneath the surface layer, the cells were grouped within lacunar spaces in clusters of from two to fourteen cells. Many of the cells were in rows and columns. The cartilage matrix appeared homogeneous, purplish in staining reaction and showed no evidence of fibrillation. The matrix at the surface and just above the calcified layer stained slightly more intensely than in the middle zone. Just above the subchondral bone was a layer of calcified cartilage, the upper surface of which was smooth, the lower border forming an irregular line of union with the bone trabeculae below. This zone of calcification was about one-third the thickness of the entire articular cartilage. Blood vessels from the intertrabecular spaces very frequently were found within this zone. The subchondral bone consisted of thick trabeculae which enclosed small spaces containing fat, numerous capillaries and small blood vessels. Normal adult metacarpal articular cartilage and subchondral bone is illustrated in Fig. 4.

The earliest constant histological change noted was a thinning of the layer of calcified cartilage. The affinity of this layer for basic stain was decreased. On the surface of the articular cartilage one

frequently observed small light staining elevations at the margins of the beginning depressions. Most of these elevations were partially covered by fibroblasts and were composed of lightly staining matrix which enclosed scattered and distorted groups of cartilage cells. In many instances, the surface cells of these elevations closely simulated the connective tissue (perichondrium) seen at the margins of the articular cartilage (Fig. 15). Associated with the above changes, one frequently observed varying degrees of fibrillation of the cartilage matrix. In its earliest stages this was shown by a basic stain-streaking of the matrix between columns of cartilage cells. The streaking and later fibrillation of the hyaline matrix was almost always in the vertical axis (see Fig. 16). Varying degrees of distortion of the rows and columns of cartilage cells were usually associated with the fibrillation. In a few instances actual vertical splitting of the matrix had occurred. Examination of blood vessels in the subchondral bone beneath these early lesions showed no constant changes. In some instances, they were dilated and engorged; in other instances they appeared deficient in number. As a result of the thinning of the calcified cartilage layer, the subchondral blood vessels were nearer to the articular cartilage proper. It should be emphasized that we never found important inflammatory cell infiltration or necrotic foci in the subchondral bone or marrow spaces.

The small, hard, yellowish elevations noted on the articular surface, when examined histologically, were found to be enlarged overgrowths of articular cartilage such as have already been described (see Fig. 16). Some of these localized outgrowths had as a result of pressure spread out to form overlapping "mushroom-like" margins. The cartilage cells in these elevations were distorted, the matrix was fibrillated and vertical clefts had formed. In some of these protuberances lime salt deposition was observed.

In slightly more advanced lesions, shallow depressions and definite thinning of the articular cartilage had occurred. The calcified zone was markedly thinned, distorted and absent in many places. The surface of these altered areas of cartilage was covered by several layers of cells which could not be distinguished from fibroblasts (see Fig. 17). In more advanced lesions, the calcified layer of cartilage had completely disappeared. All of the remaining cartilage appeared as fibrocartilage and had been invaded by blood vessels from the intertrabecular spaces of the subchondral bone (see Fig. 18). Thus,

one may state that complete degeneration of articular cartilage and repair by granulation tissue does not necessarily precede vascularization of cartilage. The disappearance of the layer of calcified cartilage appeared to be of fundamental importance.

In the medium sized lesions, as illustrated grossly in Fig. 9, the articular cartilage had completely disappeared in the sharply demarcated area of degeneration. Subchondral bone had disappeared for a depth of from three to five times the thickness of articular cartilage. At the margins of the defects there was an abrupt change from normal hyaline cartilage into fibrocartilage and fibrous tissue. This change was first apparent on the articular surface and only later in the depth of the cartilage. The bases of the defects were lined by fibrous tissue in which fairly numerous small blood vessels were seen. The underlying bone trabeculae showed evidence of atrophy and rearrangement, but no evidence of osteoclasis. The marrow spaces were filled largely with fat but showed no fibrous tissue proliferation (Fig. 19).

The largest lesions studied gave little additional information. More bone trabeculae had been resorbed so that the subchondral bone defects were six times the thickness of articular cartilage. The margins were sharp and sometimes overhanging (Fig. 20). In one instance, definite crushing of the bone trabeculae beneath the articular cartilage was observed. In occasional sections, the connective tissue lining the areas of cartilage degeneration had become very much thickened, extremely vascular, and numerous fat cells had replaced the fibrous tissue (Fig. 21).

In the old cows there was more hyalinization of the connective tissue which lined the depressed lesions. The adjacent cartilage matrix was often more intensely stained with basic dye (Fig. 22).

No important pathology was found in the associated synovial membranes. There were numerous branching small blood vessels in the subsynovial tissue, many of which showed thickened hyalinized walls. There was also a varying degree of chronic inflammatory cell infiltration in parts of the synovial membrane and subsynovial tissues. This feature, however, was never very marked. No pannus formation or synovial villi were observed in any of the joints examined.

Comment

Relatively few studies of the pathology of spontaneous joint diseases of animals have been reported. Such observations have been recorded for the most part in publications which do not readily come to the attention of those interested in the study of human arthritis. The value of an intensive study of arthritis in domestic animals is well illustrated by the work of Hare [3] who, from a careful and extensive pathological study of rheumatoid arthritis in horses (the human proliferative type of Nichols and Richardson [4]), was able to describe in detail the changes in all portions of the involved joints. While areas of degeneration on opposing cartilage surfaces were observed by Hare, they were always associated with other important joint changes which were thought by him to follow inflammatory processes in the vascular connective tissue of the joints and tendons.

Chronic arthritis specifically involving the carpal articulation of horses has been described by Cherry [5] and Krüger.[6] Although Krüger mentioned areas of degeneration of cartilage on opposing articular surfaces, there were other associated articular changes. These changes were lipping, osteophyte formation, proliferation and inflammation of the synovial membrane.

In papers dealing with the disease process termed spavin of the tarsometatarsal articulation of horses and cattle, one finds an occasional brief description of pathological changes comparable to those that are the subject of this paper. However, the process appears to have been entirely different in that various workers [7, 8, 9] have directed attention to the inflammatory nature of the disease and the tendency to ankylosis of the involved joints.

Lesions such as are described in the present study are not mentioned by Hutyra and Marek [10] who, in discussing articular rheumatism of domestic animals, stated that it was most frequently seen in cattle. In cattle this disease affected delicate milkers most commonly, less often oxen, and was scarcely ever encountered in grazing cattle. It was assumed that the disease described (articular rheumatism) was due largely to bacterial infection.

The present investigation deals with progressive lesions of the articular cartilage of cattle which occur on the opposing articular surfaces of the medial side of the metacarpal and carpal bones. These

lesions were self-limited and not accompanied by any important pathological changes in other portions of the involved joints.

Possible etiological factors concerned with the degenerative lesions of the carpometacarpal articulations have been considered. The gross and microscopic examination showed that they were of a degenerative nature. No lesions were ever found in the carpometacarpal joints of calves slaughtered at the age customarily used for veal. The earliest lesions were found in young beef cattle (under 2 years of age), and the more extensive lesions occurred in some of the older animals. It is important to emphasize that the lesions did not necessarily progress once they had developed. Having reached their maximum size, they remained as depressed areas with little or no attempt to repair.

The degenerative changes in articular cartilage appeared to be unrelated to vascular changes as factors of causation. From the study of embryo and calf specimens it was observed that the blood supply to both the medial and lateral articular cartilage disappeared at the same time, yet the areas of degeneration occurred only on the medial cartilage. These blood vessels had all disappeared in the beef cattle before the age of 2 years. Examination of the blood vessels in the subchondral bone beneath the developing defects in cartilage did not reveal any abnormalities which were constant or of histological importance. The arterioles in the fibrous tissue lining the areas of degeneration (once formed) were often thick-walled and hyalinized. This factor was thought to be secondary to the alterations already present rather than to be in any way responsible for them.

Bacterial infection may be dismissed as a causative factor because of the constant occurrence of this lesion in all animals, the constant involvement of one localized area of the articulation, and because there is no histological or cytological evidence of infection to be found in the synovial membranes, articular cartilage, subchondral bone, or synovial fluid.

Gout as an etiological factor was ruled out since deposits of sodium urate crystals never were found upon articular surfaces or in the articular cartilage of either early or late joint lesions.

The constant occurrence of the localized articular cartilage degeneration in young western beef cattle and older milch cows * ap-

* These cattle were obtained largely from the New England states.

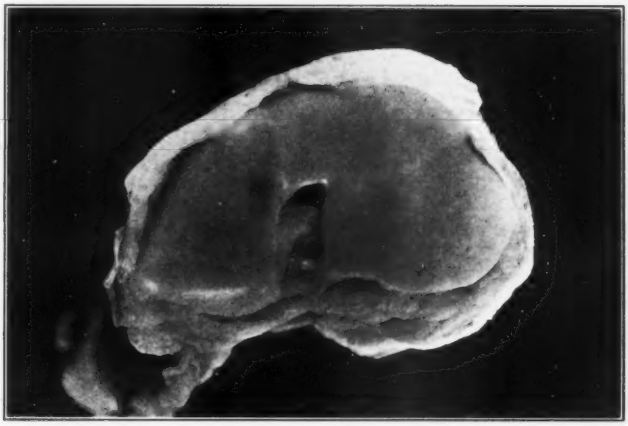
pears to rule out differences of activity and habitat as being of importance in causation.

The study of embryo joints failed to reveal anything unusual in the development of the carpometacarpal joints. It was learned from these specimens, however, that the articular cartilage rapidly decreased in thickness in the early months of life. When large transverse sections of the metacarpal articular cartilage and subchondral bone of embryos, calves and cattle were mounted in series on large glass plates and studied with a hand lens, interesting structural changes in the subchondral bone were noted. In the embryos and calves, the subchondral bone trabeculae were evenly distributed beneath the articular cartilage (Fig. 23). In the older animals one noted a rearrangement of the trabeculae and an increase in the thickness of the bone cortex. There was a marked difference in the manner in which the compact cortical bone expanded into the subchondral bone trabeculae of the medial and lateral sides (Fig. 24). The lateral cortex of the metacarpal bone flared sharply from the vertical axis, so that the vertical subchondral bone trabeculae supported adequately the entire lateral articulating surface, whereas the vertical subchondral bone trabeculae branching from the mesial cortex supported only the medial one-half of the medial articular surface. The central area of the entire articular surface was well supported by the dense bone which resulted from the fusion of two metacarpal bones. This rearrangement of bone trabeculae resulted in an area of rarefied subchondral bone which was directly beneath the site of these cartilage defects. These areas of rarefied subchondral bone were continuous with the marrow spaces of the bone metaphysis. The fact that the articular cartilage defects occurred directly over these areas of rarefied bone suggests that they are potential weak spots and therefore of great etiological importance. Such a deduction is further substantiated by the fact that the few remaining bone trabeculae underlying the larger lesions had been so arranged that they paralleled the surface cartilage. The latter structural arrangement was apparently a compensating phenomenon.

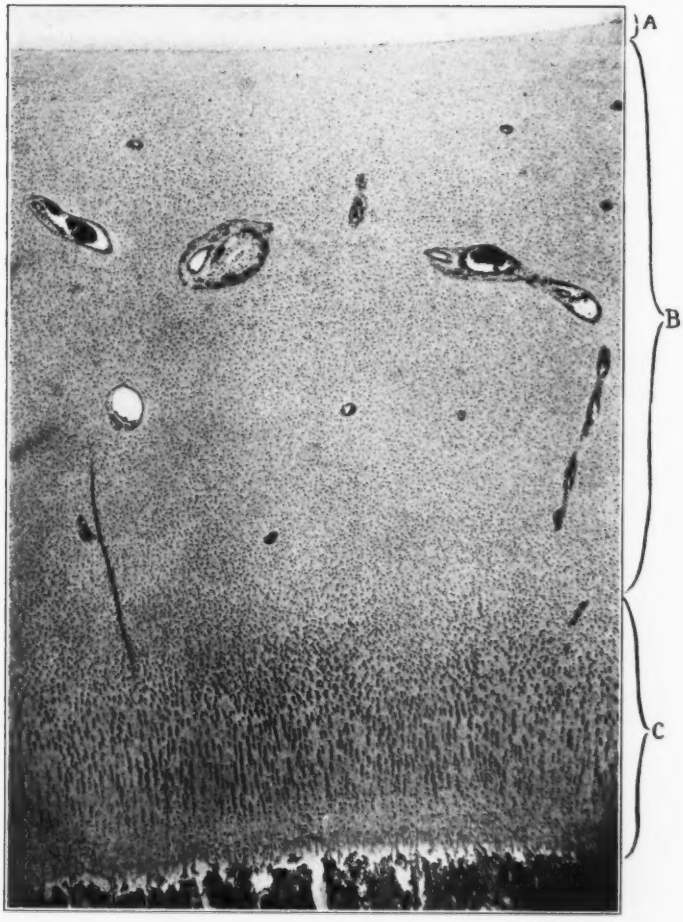
The gross structure of the carpometacarpal articulation of the cow seemed equally as important etiologically as the structural rearrangement of the subchondral bone. For this reason, the corresponding joints of the horse were incorporated in this study. Such specimens were not similarly involved even though the majority of them were

the seat of marked arthritic changes. From the mechanical standpoint, the carpometacarpal joint of the horse is more nearly perfect than is the corresponding joint of the cow. In the case of the former animal there are more articulating concavities and convexities which would aid in preventing anteroposterior or lateral slipping and the posterior portion of the joint is strengthened by two very strong articular ligaments. The metacarpal articulating surface of the cow is comprised of two large flat surfaces which are divided by a single narrow anteroposterior ridge situated just lateral to the midline. Only one articular ligament connects the carpal and metacarpal bones in cattle. In the cow one finds a considerable degree of genu valgum of the fore leg. Because of this factor, it would appear that the greatest weight is borne on the medial aspect of the carpometacarpal joint. Repeated traumatic injury applied to the articular surfaces of a weakly constructed joint might well explain the lesion in question, particularly since such lesions occur regularly over the area of least bony support. A probable source of repeated trauma is found in the manner in which a cow uses the front knees (carpometacarpal articulations) in the process of lying down and getting onto her feet from the recumbent position. In lying down, the cow drops the weight of the fore quarters upon one sharply flexed carpometacarpal joint and then upon the other. In rising, the front legs are folded under the thorax and the weight of the fore quarters of the animal is carried on the front knees until the hind quarters are upright, when with great exertion, the fore quarters are lifted from one front knee at a time. This series of movements is entirely different from those of the horse where the fore legs are extended forward and the weight of the fore quarters lifted onto the fully extended fore legs with the aid of forceful pushing by the rear extremities. If the above observations are indicative of the stresses applied to the carpal and metacarpal bones of the cow, then these articular cartilage lesions may well be considered as traumatic in origin.

It has been previously stated that the degeneration involved similarly the two opposing cartilage surfaces. This was true in both the early and the more extensive lesions. While this fact is apparently in agreement with a theory of traumatic origin, it does not explain why the two opposing depressed areas extended deeper, once formed. This phenomenon may be explained as follows: Once the lesions are formed, the weight is carried on the surrounding intact articular sur-



1

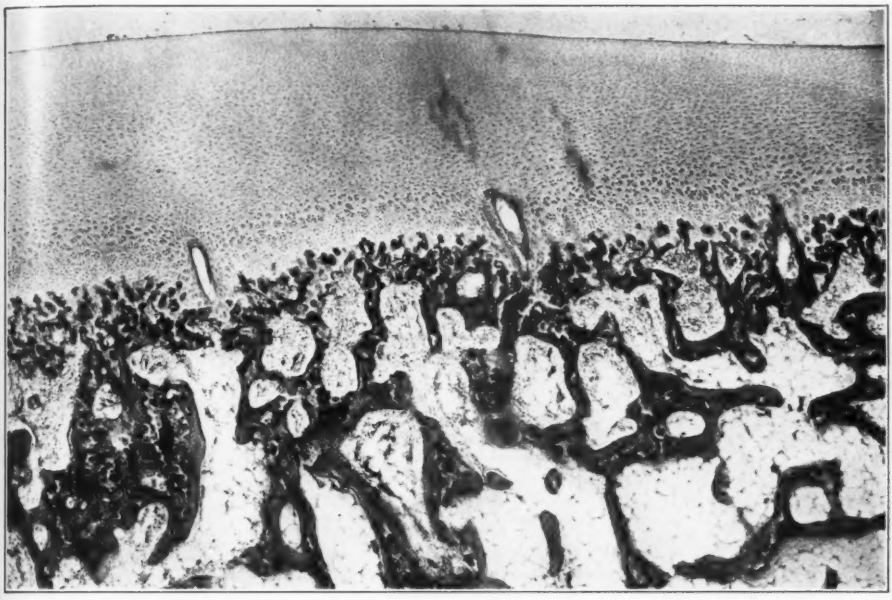


2

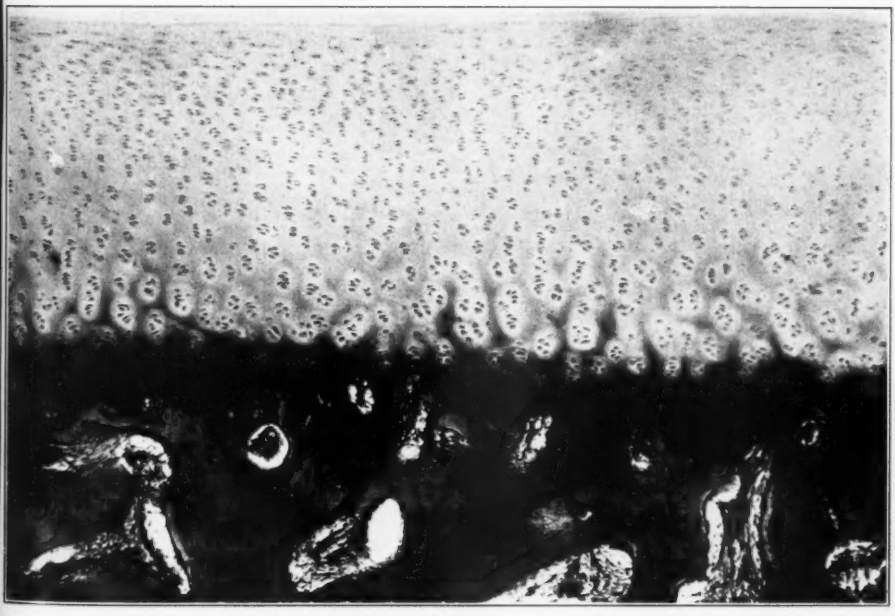
PLATE 71

FIG. 3. A low power photomicrograph of the metacarpal articular cartilage of a calf. Note the reduced thickness of cartilage (as compared to Fig. 2), the prominent perichondrial border, and the incorporation of three blood vessels by subchondral bone growth. The layer of provisional calcification is well formed. × 37.

FIG. 4. A photomicrograph of a normal portion of adult articular cartilage of the cow. One should note the orderly arrangement of cells in lacunar spaces, and the wide zone of calcified cartilage. The subchondral bone trabeculae are broad and very dense. × 84.



3



4

Bennett and Bauer Degeneration of Cartilage in Bovine Joints

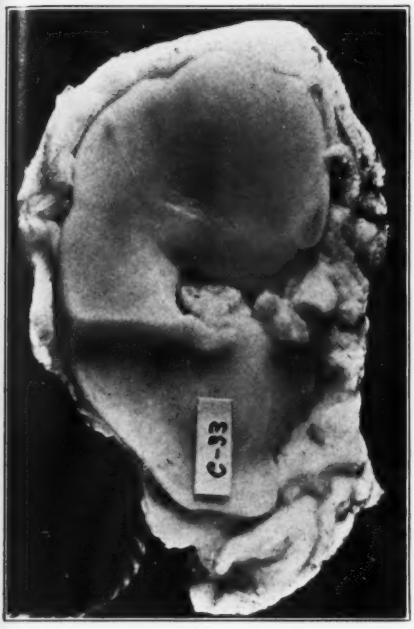
PLATE 72

FIG. 5. A metacarpal articular cartilage of a young steer (under 2 years of age). Note the early roughening of articular cartilage in the center of the medial surface. Natural size.

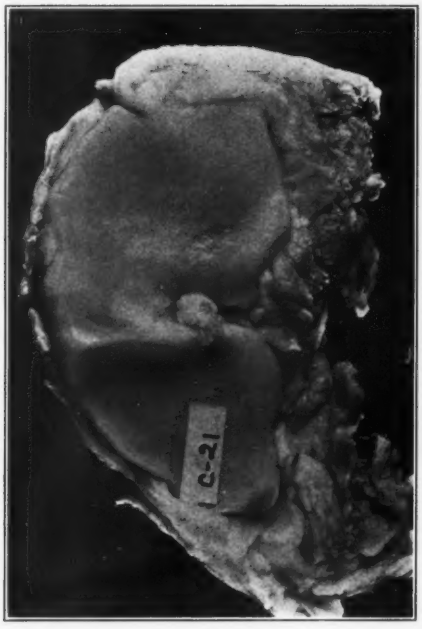
FIG. 6. The medial articular cartilage of this joint shows an area of roughening and thinning which is slightly larger than the defect in Fig. 5. Natural size.

FIG. 7. The central area of the medial articular cartilage is more depressed and the cartilage has been more completely destroyed than in the earlier illustrations. Natural size photograph.

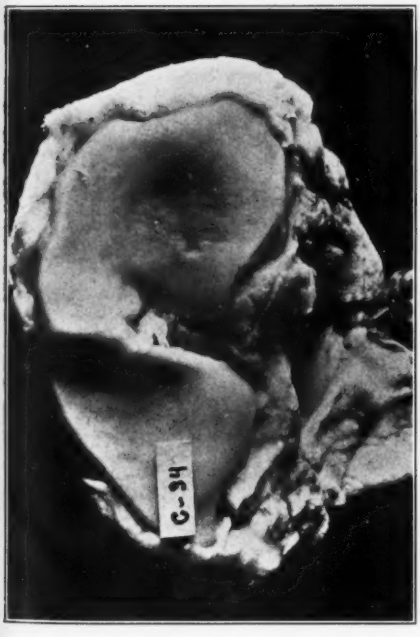
FIG. 8. A natural size photograph of a slightly larger area of degeneration in articular cartilage. This lesion extended down to subchondral bone in a few areas.



5



6



7

8

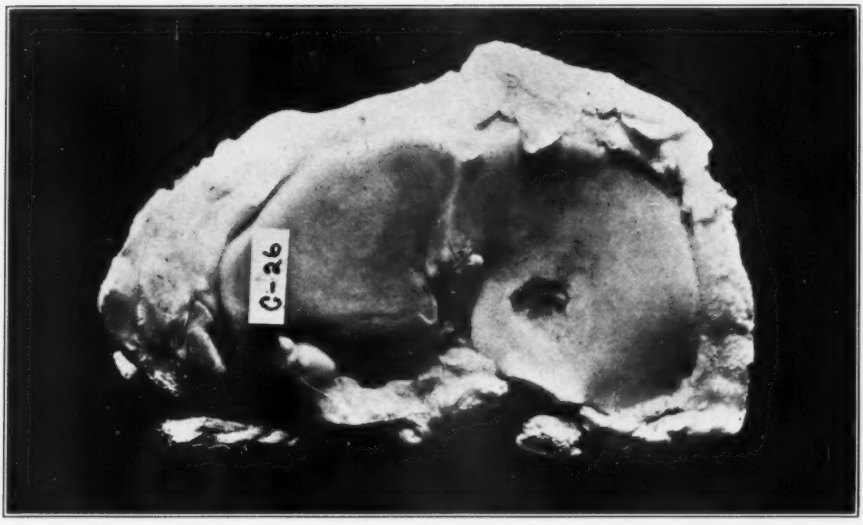
Bennett and Bauer

Degeneration of Cartilage in Bovine Joints

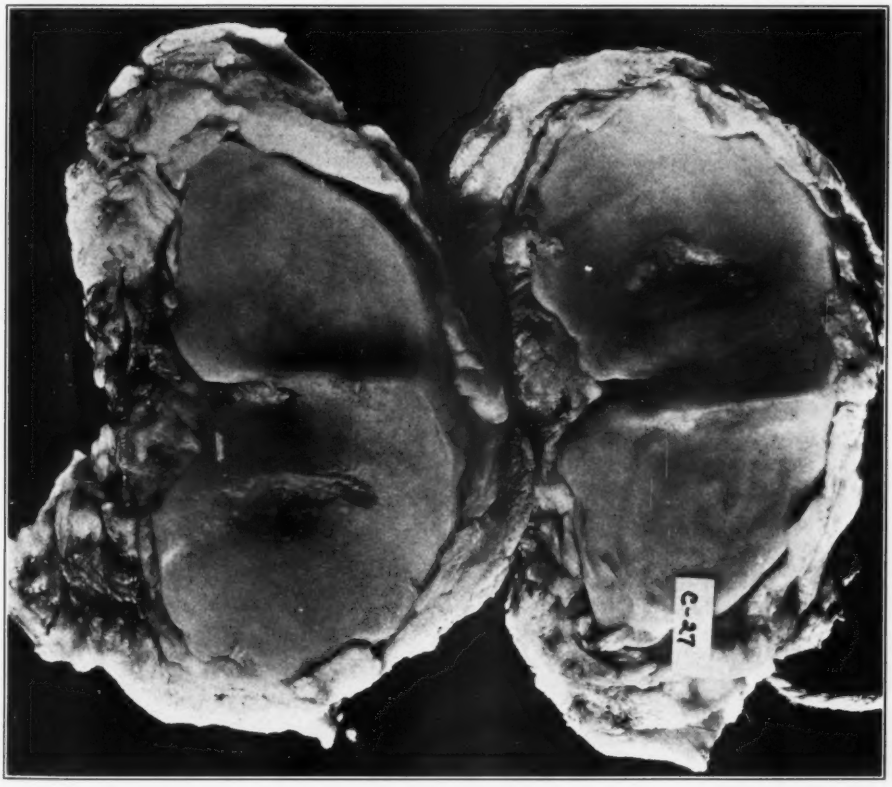
PLATE 73

FIG. 9. A natural sized photograph showing an average sized lesion in articular cartilage which extended into subchondral bone. Note the sharp overhanging margins.

FIG. 10. The articular cartilage surfaces of the right and left metacarpal bones of the same steer. Note the similarity in size, shape and location of the degenerative lesions. Natural size.



9



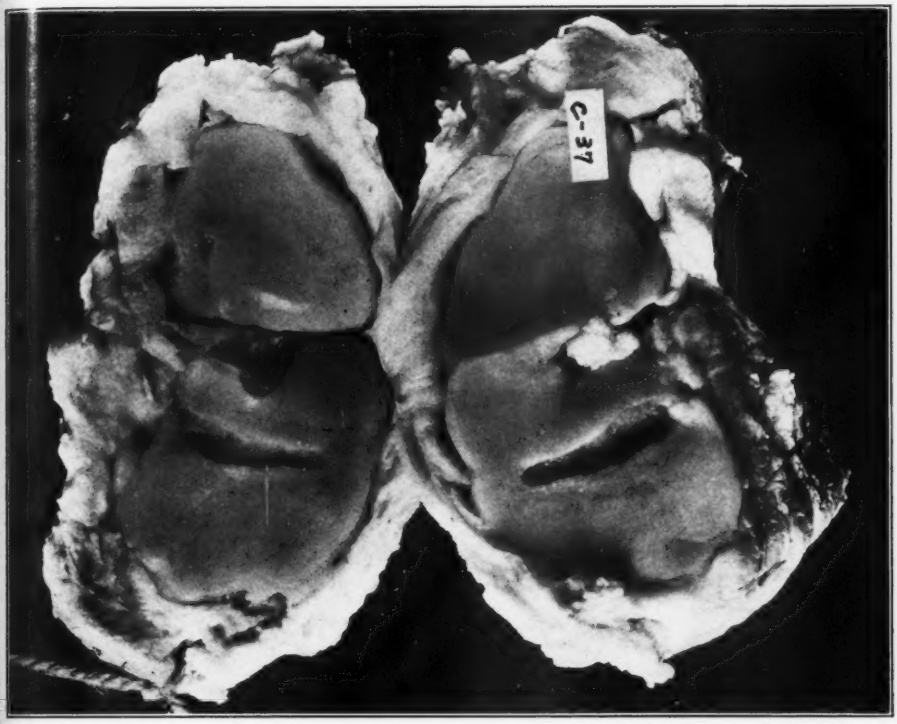
10

Bennett and Bauer Degeneration of Cartilage in Bovine Joints

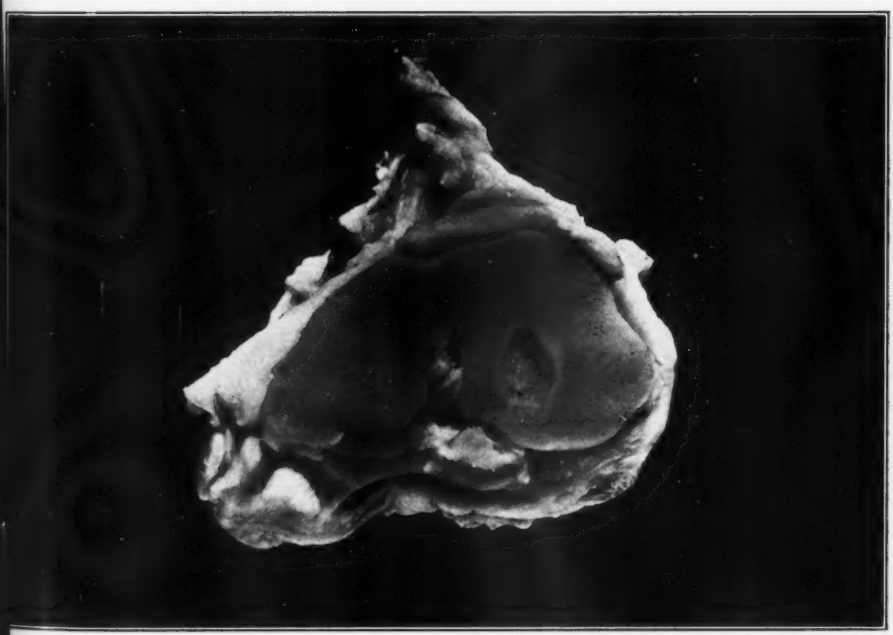
PLATE 74

FIG. 11. A carpometacarpal articulation opened in such a way as to show the opposing carpal and metacarpal articular surfaces. The similarity of size and type of degenerative lesion is apparent. Natural size.

FIG. 12. An average sized area of degeneration in cartilage and subchondral bone of an old milch cow. Note the light marginal halo produced by a yellow coloration of the articular cartilage at the margin of the lesion. This feature is peculiar to the lesions in older cattle. Natural size.



11

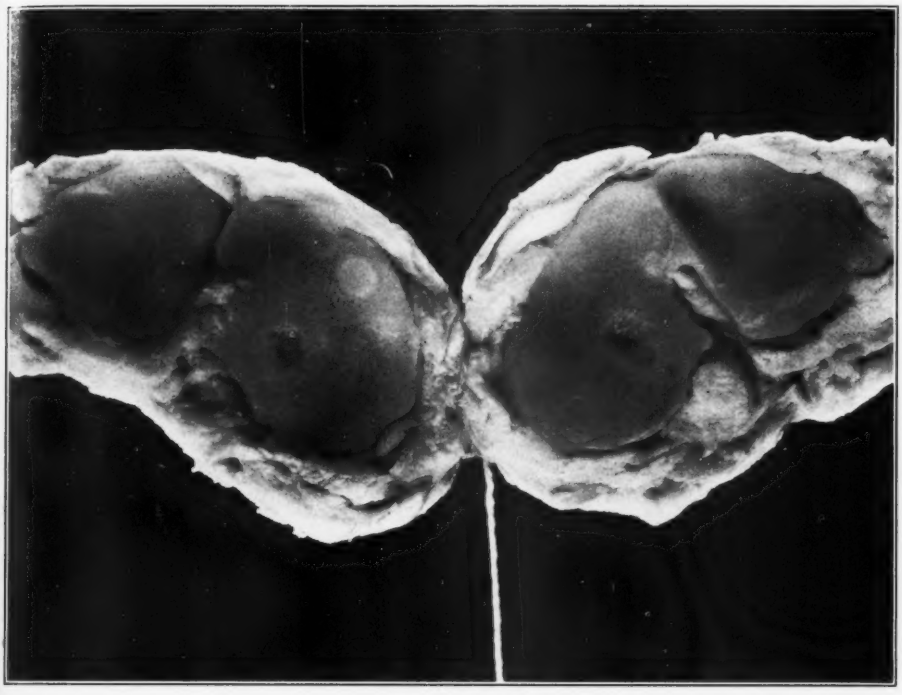


12

Plate 75

Fig. 13. Similar lesions on corresponding surfaces of the carpal and metacarpal articular cartilages of an old milch cow. Natural size.

Fig. 14. The articular surfaces of the carpal and metacarpal bones of an old milch cow, showing the striking similarity of the lesion on opposing surfaces. Natural size.



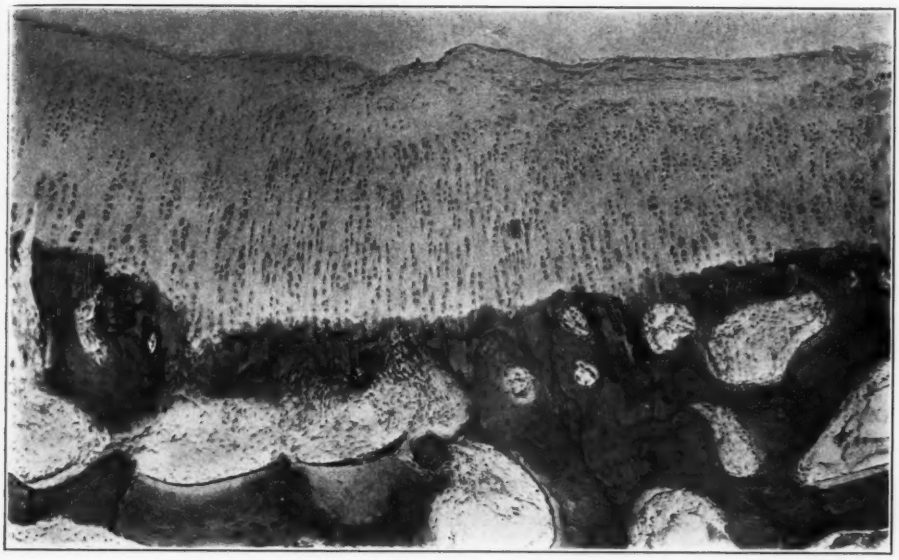
13

14

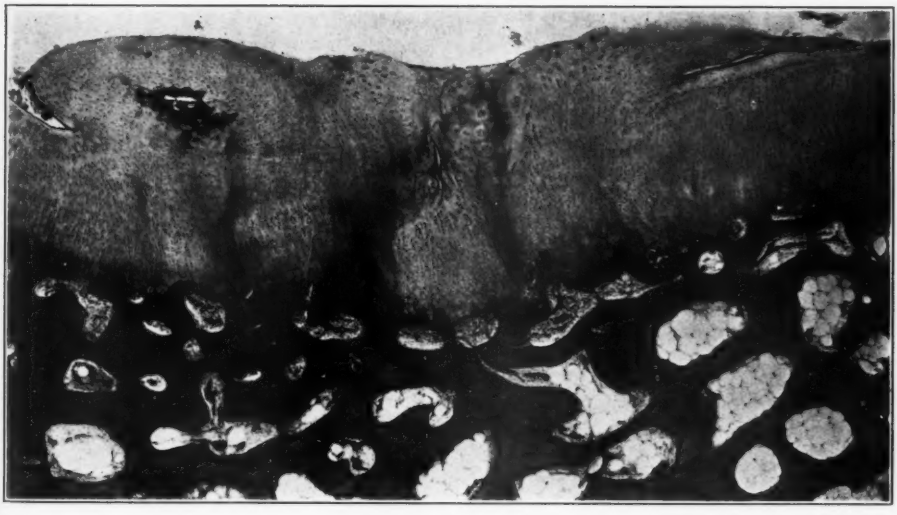
PLATE 76

FIG. 15. Early changes in articular cartilage are illustrated in this photomicrograph. The layer of calcified cartilage is thinned out, slightly depressed and completely broken in many places. Beginning distortion of cartilage cells and a surface proliferation of cartilage is illustrated. × 91.

FIG. 16. A very low power photomicrograph showing more extensive distortion of cartilage and proliferation of surface cells. There is much fibrillation and splitting of the articular cartilage matrix. One area of calcification has occurred. Note the depression, thinning and disruption of the calcified layer of cartilage. × 33.



15

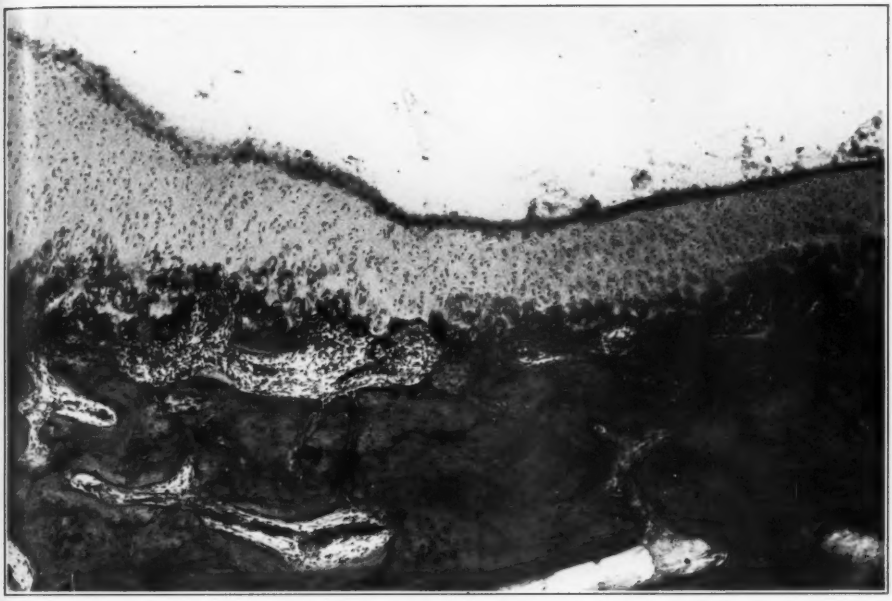


16

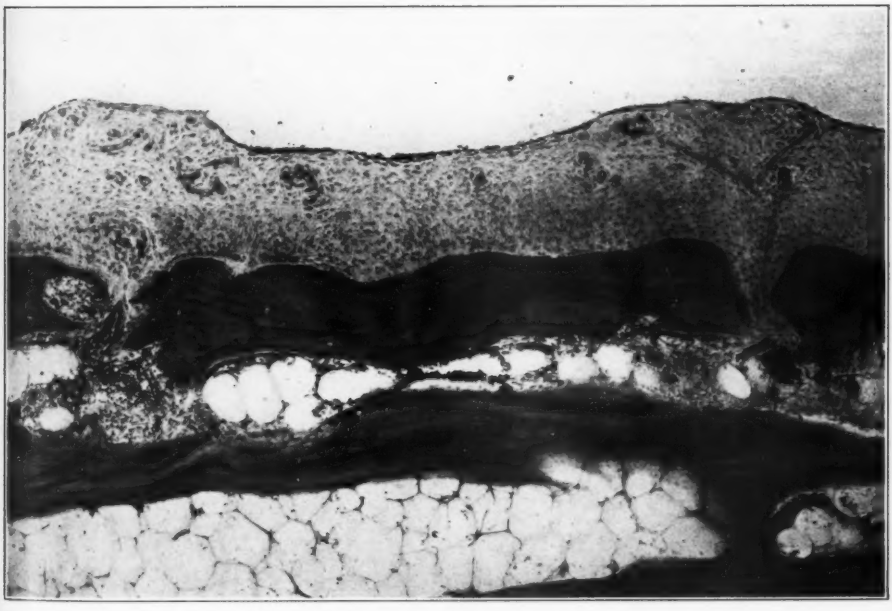
PLATE 77

FIG. 17. Beginning depression of articular cartilage and nearly complete destruction of the layer of calcified cartilage is illustrated in this photomicrograph. Note the alteration of cartilage and its resemblance to fibrocartilage. × 91.

FIG. 18. A more advanced metaplasia of cartilage into fibrocartilage. Note the complete absence of the calcified layer and the invasion of cartilage by blood vessels from the subchondral bone. × 91.



17

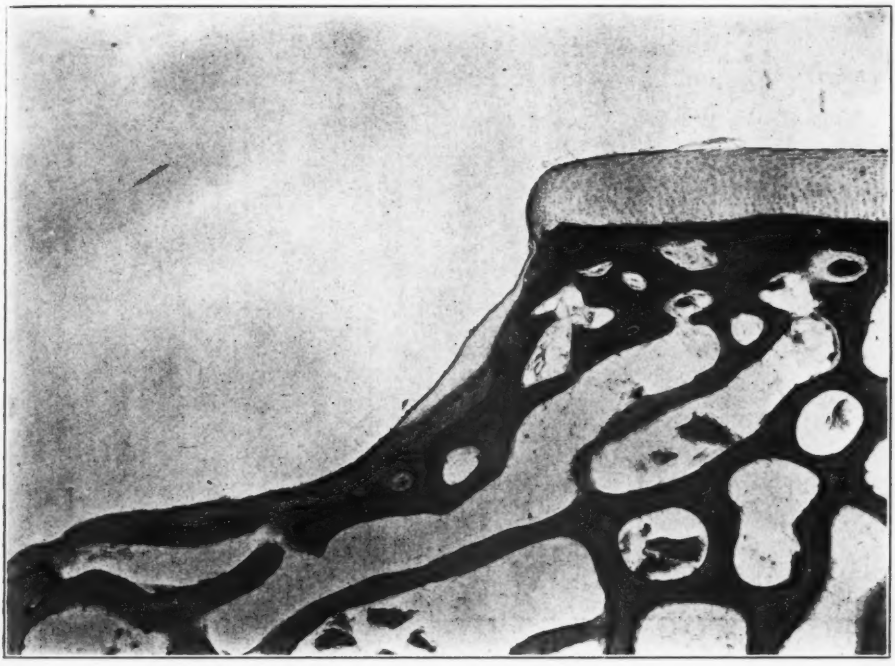


18

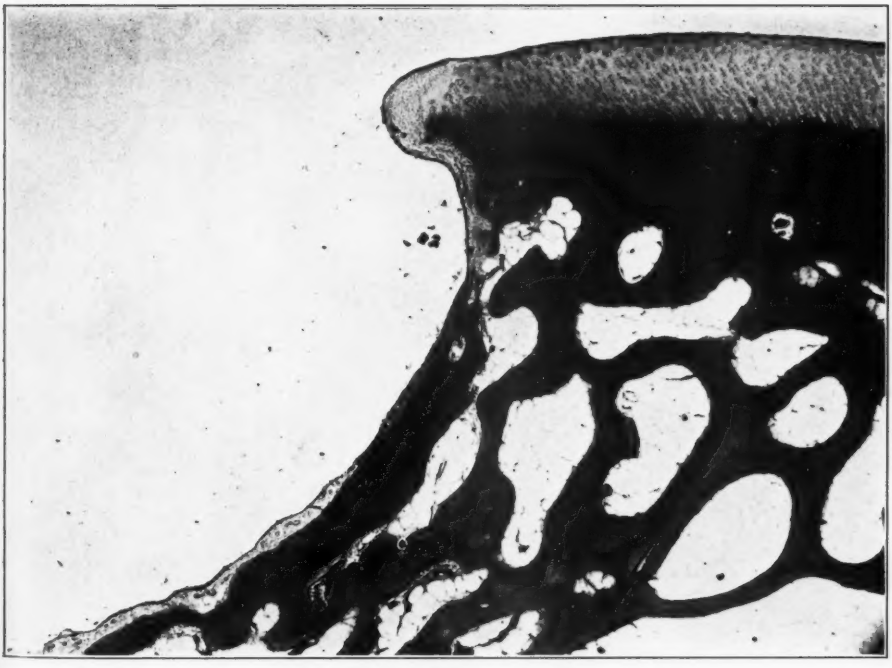
Bennett and Bauer Degeneration of Cartilage in Bovine Joints

PLATE 78

FIGS. 19 and 20. Low power photomicrographs which include one-half of each of the larger lesions from two specimens. Note the abrupt break in articular cartilage, the deep extension of the lesions into subchondral bone and the synovial membrane-like tissue lining the depressions. There is practically no inflammatory cell infiltration in any part of the sections. × 36.



19

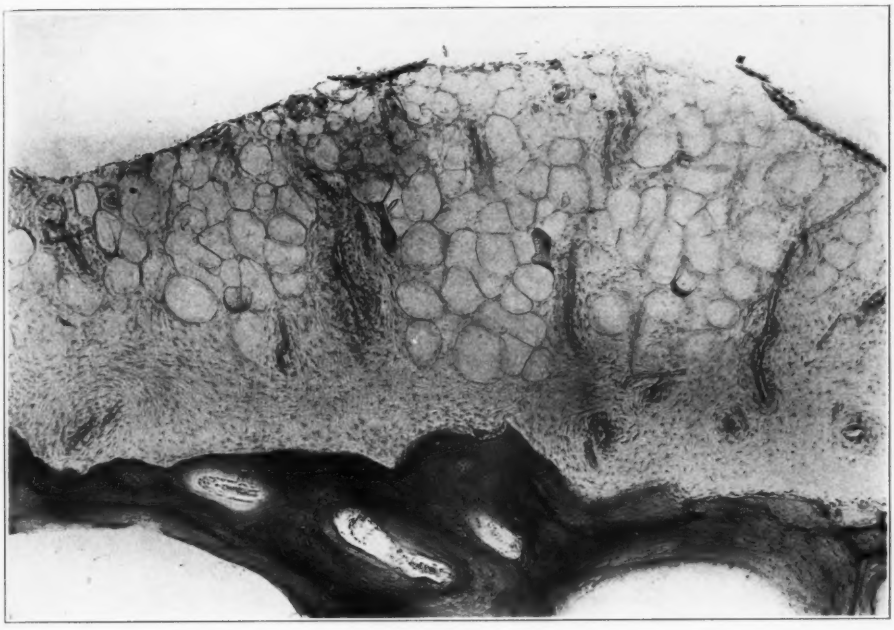


20

PLATE 79

FIG. 21. Fat replacement of the connective tissue and fibrocartilage which lined the larger and more extensive defects of articular cartilage is illustrated in this photomicrograph. × 91.

FIG. 22. One margin of a degenerative lesion in the articular cartilage of an old milch cow. Note the decrease in number of cartilage cells in the more intact cartilage and the intense calcification of the deepest layer of cartilage. The connective tissue lining the degenerated area is largely hyalinized. × 91.



21

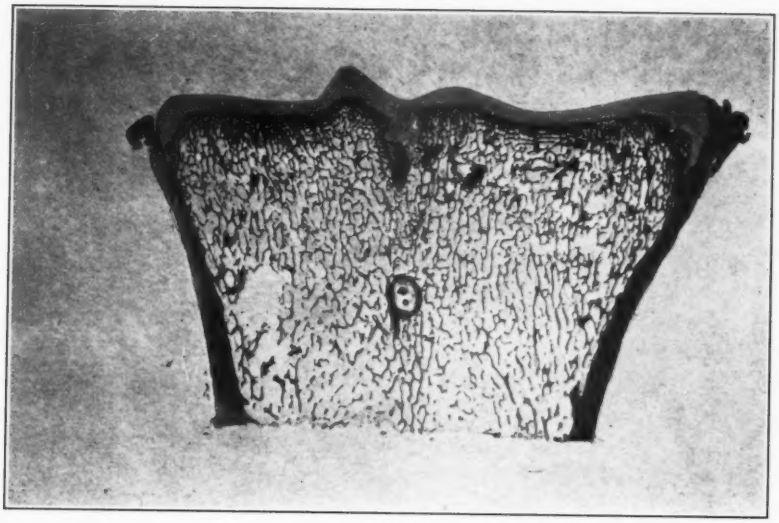
22

Bennett and Bauer Degeneration of Cartilage in Bovine Joints

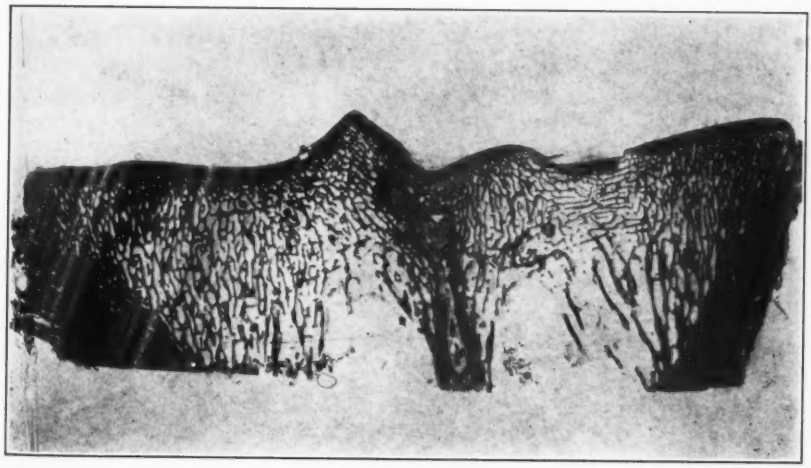
PLATE 80

FIG. 23. A photograph of an entire transverse section (celloidin) showing the evenly distributed subchondral bone trabeculae and thin cortex of the metacarpal bone of a calf. × 2.

FIG. 24. A photograph of an entire transverse section (celloidin) of the metacarpal articular cartilage and subchondral bone from a young steer with an average sized lesion of articular cartilage. The difference in the arrangement of the bone trabeculae beneath the medial and lateral articular surfaces and the cartilage defect is clearly shown. × 2.



23



24

face, where the underlying bone is more directly continuous with the solid cortical shaft. Atrophy and resorption of the bone underlying the cartilage lesions might well take place because the pressure stimulus had been removed.

The histological and gross study showed that the lesions under discussion were not the same as the pathological changes described in any type of human arthritis. The fact that these lesions began as areas of articular cartilage degeneration makes the process more nearly comparable to the degenerative arthritis of Nichols and Richardson [4] than to the proliferative type described by them. They described the degeneration of articular cartilage on one joint surface and a compensatory overgrowth of cartilage and later bone on the opposing joint surface. This compensatory overgrowth of cartilage allowed continued apposition of the involved joint surfaces. In the lesion found in the cow, the degeneration occurred on opposing areas so that continued apposition of the defects was not possible. Nichols and Richardson described thickening and eburnation of the underlying bone. Such changes were never encountered in the joints of cows under study. The degenerative type of arthritis is a disease of older individuals and more common in women. Sex and age (with the exception of the necessary first two years of life) certainly play no part in the incidence of this disease in cattle.

Following the thinning of articular cartilage and rearrangement of bone trabeculae of the metacarpal bones in young cattle, degenerative lesions developed. The sequence of pathological changes leading to degeneration of cartilage appeared to begin with thinning and disruption of the layer of calcified cartilage. Fibrillation of the cartilage matrix followed and was accompanied by replacement of the surface articular cartilage with connective tissue. Disappearance and distortion of cartilage cells was then noted, together with more marked fibrillation of the articular cartilage matrix. Splitting of the fibrillated matrix followed in some instances. Blood vessels grew into the altered cartilage through gaps in the calcified zone, entering from the intertrabecular spaces of the subchondral bone. The altered articular cartilage eventually completely disappeared and varying degrees of subchondral bone resorption followed. The cartilage and bone defects became lined with a vascular connective tissue which resembled synovial membrane to a certain extent.

Summary

1. Constant differences in the synovial fluid of the carpometacarpal and astragalotibial articulations of the cow have been described in a previous publication.[1] The finding of areas of degeneration in the articular cartilages of the carpometacarpal articulations of all cattle over 2 years of age would appear to be an adequate explanation of the synovial fluid differences observed.

2. These areas of progressive degeneration in articular cartilage have been studied systematically and the successive changes have been described and illustrated.

3. The development of the carpometacarpal articulations was studied in a series of bovine embryos and calves. The vascular articular cartilages of embryos and calves became avascular before the animals attained the age of 2 years. Pronounced rearrangement of the subchondral bone trabeculae resulted in a relatively deficient bony support of the medial articular cartilage where the degenerative lesions occur.

4. The possible etiological factors of such cartilage lesions have been discussed. It was concluded that they were probably due to repeated trauma in weakly constructed articulations. Deficient subchondral bone support was thought to be an important predisposing factor.

5. The type of cartilage lesion described in this paper is not wholly similar to any of the joint lesions described in human arthritis.

Note: We wish to thank the New England Dressed Meat and Wool Company for the material used in this study.

REFERENCES

1. Bauer, Walter, Bennett, G. A., Marble, Alexander, and Claflin, Dorothy. Observations on normal synovial fluid of cattle. I. The cellular constituents and nitrogen contents. *J. Exper. Med.*, 1930, **52**, 835.
2. Mallory, F. B., and Wright, J. H. Pathological Technique. W. B. Saunders & Co., Philadelphia, 1924, Ed. 8, 54–55.
3. Hare, Tom. An investigation of the etiology and pathogeny of equine chronic arthritis (rheumatoid arthritis). *Vet. Rec.*, 1927, **7**, 411.
4. Nichols, E. H., and Richardson, F. L. Arthritis deformans. *J. Med. Res.*, 1909, **16**, 149.
5. Cherry, W. A. On carpitis. *Veterinarian*, 1845 (Nov.), **18**, 601.
6. Krüger. Die chronische Arthritis und Periarthritis Carpi des Pferdes. *Arch. f. wissensch. u. prakt. Tierh.*, 1905–06, **32**, 391.
7. Smith, F. Some joint diseases of the horse. *J. Comp. Path. & Therap.*, 1893, **6**, 149.
8. Cadéac, M. C. Dry arthritis of the hock. *J. Comp. Path. & Therap.*, 1909, **22**, 41 and 97.
9. Goldberg, S. A. The pathology of spavin. *J. Med. Res.*, 1918, **33**, 225.
10. Hutyra, Franz, and Marek, Joseph. Special Pathology and Therapeutics of the Diseases of Domestic Animals. A. Eger, Chicago, 1916, **2**, 873.

DESCRIPTION OF PLATES

PLATE 70

FIG. 1. A natural size photograph showing the normal metacarpal articular cartilage of a young calf.

FIG. 2. A photomicrograph of very low magnification showing the entire thickness of cartilage of a 59 cm. bovine embryo. Note the three merging zones of cartilage: (*a*) superficial or perichondrial; (*b*) middle or vascular; (*c*) deep or expanding. × 32.

METASTATIC CARCINOMA SIMULATING HYPERPARATHYROIDISM *

R. L. Mason, M.D., and Shields Warren, M.D.
(From the Lahey Clinic and the Laboratory of Pathology, New England Deaconess Hospital, Boston, Mass.)

Upon the basis of experimental and clinical data developed after the discovery of parathormone by Collip,[1] there has been evolved the conception of a clinical syndrome of parathyroid hyperfunction, or hyperparathyroidism. The diagnosis has come to be based upon the following criteria: (1) increased blood calcium, (2) lowered serum phosphorus, (3) increased calcium excretion, (4) widespread rarefaction of bones, and (5) presence of a parathyroid tumor.

Important symptoms are muscular weakness, nausea and vomiting, polyuria and polydipsia, and renal colic.

All of the criteria mentioned have not been present in every reported case; hypercalcemia has been the only constant finding. Thus, in the case reported by Richardson, Aub and Bauer [2] a parathyroid tumor was not found. In Pemberton and Geddie's case[3] there were no demonstrable bony changes. In Wilder's case [4] the calcium excretion was not increased although this may have been influenced by diet and ultraviolet radiation. Barr and Bulger [5] and Hunter [6] present extensive bibliographies which need not be repeated here. Accompanying both articles are abstracts of cases reported up to the past few months.

In the case we wish to report, the interest lies in the decision as to whether we were dealing with a true case of hyperparathyroidism or a simulated condition.

Case Report

Clinical History: The patient, a woman 47 years of age, consulted the Clinic on October 28, 1929, complaining of generalized pains, weakness and vomiting of ten months' duration. The family history was not significant. She had had no infectious diseases. There was nothing to suggest luetic infection. Five years before, a tumor of the left breast had been removed. She had been told at that time that "it might have caused trouble had it not been removed." †

* Received for publication May 9, 1931.

† A report from the hospital where the operation was performed gave the diagnosis as carcinoma. No microscopic report was obtained.

In general she considered herself to be in good health up to the present illness which had been gradual in onset, starting ten months before. She had been confined to bed for the preceding six weeks. At the onset she was troubled with small areas of soreness and twinges of pain in the anterior thoracic wall.

The ribs were sensitive to pressure and any muscular effort, such as deep breathing or using the pectoral muscles, caused sharp pain. After a time, similar aches and pains appeared in the arms, legs and back. These occasioned much suffering, especially with motion. After a time the muscles became "weak all over" and she was unable to walk or to use her arms except with great effort. Four months before entry, following X-rays of the teeth, three were removed. This was followed by an "osteomyelitis" of the jaw, which had continued to drain. A few weeks before she had developed marked polydipsia and urinary frequency, night and day.

There was marked anorexia. During the preceding month she had vomited several times daily without any definite relation to meals. She had lost 20 pounds in weight since the onset of illness ten months before. A "goiter" had been present for several years.

Physical examination showed a thin, sallow, extremely apathetic woman looking a great deal older than her stated age of 47 years. There was evidence of considerable loss of weight. There was a partial left-sided facial paralysis and left-sided atrophy of the tongue. The musculature everywhere was extremely flabby and all movements were made with considerable effort. In the right mandible, anteriorly, at the site of extraction of two teeth, there was a sinus tract containing a packet of gauze. On removal of the gauze there was an escape of a small quantity of thin purulent material. The breath was uremic. The eye grounds were negative. The right lobe of the thyroid was occupied by a firm rounded mass 5 cm. in diameter. Examination of the heart and lungs elicited nothing significant except that there was pain on deep inspiration. There was some tenderness on pressure on the ribs, anteriorly. There was a scar from the breast operation on the left side. This apparently had been a simple mastectomy. There were no nodules in the axilla or supraclavicularly. The liver edge was palpated two fingers' breadth below the costal margin. Pelvic examination revealed what at first was thought to be a mass, but later was thought to be retroverted uterus. In the right wrist was palpated a bony mass 2 cm. in diameter, obviously arising from one of the carpal bones. All reflexes were present but sluggish. Blood pressure 140/80.

The laboratory examinations at entrance revealed the following: Urine, a very slight trace of albumin with 1 to 3 hyaline casts, 1 to 2 granular casts and 15 to 20 white blood cells per high power field. The urine was negative for Bence-Jones protein. There was a moderate secondary anemia. The white blood cells numbered 10,000. The blood non-protein nitrogen determination showed a value of 56 mg. per 100 cc. The phenolphthalein excretion was 24 per cent in two hours. Wassermann test negative. The blood calcium was 17.3 mg. per 100 cc., the blood phosphorus 4.1 mg. Blood bilirubin 0.8 mg. (van den Bergh).

Following is the report of the X-ray findings, interpreted by Dr. L. B. Morrison:

"The skull shows moderate density and shows some radiolucent areas, two that are at least 1 cm. in diameter, and several areas that are smaller. These are definitely metastatic, the adjoining bone

being of normal density. The right mandible shows an area of diminished density just in front of the first molar and down to about the level of the dental foramen.

The ribs show many minute radiolucent areas of the general process compatible with metastases. The third and fourth dorsal bodies are crushing, and they are quite dense. The trachea is crowded slightly to the left, apparently by a small thyroid.

The scapula and upper end of the right humerus show very definite radiolucent areas in which the bone is being destroyed, the adjoining bone being of normal density. The right hand shows radiolucent areas at the base of the fourth metacarpal, and in relation to the os magnum and unciform. The radius and ulna are of normal density, as is the lower end of the humerus.

The fifth lumbar body and both the sacrum and the ilia show radiolucent areas compatible with a general carcinosis. The liver is becoming slightly enlarged. The lungs show no particular changes. The right femur and tibia show no definite changes. The bone is of normal density."

As seen from the above report, Dr. Morrison considered the bone changes compatible with generalized bone metastases. Moreover, he felt that if the condition was due to hyperparathyroidism, there should be no areas of definitely normal bone without calcium deficiency, as there were here.

During the subsequent period of observation the patient's general condition improved somewhat. Vomiting, at first frequent, was finally controlled by intravenous glucose and by careful diet. Under this treatment, also, the blood non-protein nitrogen became lower and the phthalein excretion greater. Anorexia continued, as did the marked weakness and apathy. The high blood calcium determination was checked and persisted at a high level (Table I). The blood phosphorus continued at a normal level. Albright, Bauer, Ropes and Aub,[7] in studying the phosphorus level in the blood, found that parathormone primarily lowers the phosphorus level. If, however, the serum calcium runs above a critical level of about 14 to 15 mg. per 100 cc., the urinary phosphorus excretion falls and the blood phosphorus rises. This may account for the high blood phosphorus levels in this case.

During the following week studies were made of the calcium excretion. A low calcium diet was given (see Table II). After three

days, all urine and feces were saved for a three-day period for calcium determination. The data are shown in Table III. A negative calcium balance of 139 mg. or 45 per cent was revealed. Bauer, Al-

TABLE I

Serum Calcium and Phosphorus *

Date	Blood calcium	Blood phosphorus
	mg.	*mg.*
Nov. 4	17.3	4.1
" 5	17.6	..
" 11	16.6	4.0
" 15	15.0	3.0
Operation		
Nov. 16	..	..
" 17	13.8	3.3
" 18	13.7	3.2
" 19	13.4	..
" 20	13.7	2.5
" 21	13.7	2.5
" 24	12.9	2.9
Dec. 2	14.1	3.1

* Determinations by Miss H. M. Hunt, New England Deaconess Hospital.

TABLE II

Phosphorus and Calcium Intake

Date	Calcium	Phosphorus
	mg.	*mg.*
Nov. 8	99	169
" 9	91	120
" 10	136	532
" 11	110	149
" 12	102	172
" 13	95	125

bright and Aub,[8] however, found that when normal individuals were fed on calcium diets (100 mg. ± daily) negative calcium balances were manifested. Some of them were equal to the negative balance in this case.

Although the evidence was not conclusive, exploration for parathyroid tumor seemed warranted. Operation was done November 16. The report follows:

"Ethylene anesthesia. Usual thyroid exposure. The entire right lobe of the thyroid was occupied by a firm mass 5 cm. in diameter. It was exceedingly friable and was adherent to its bed and to the muscles laterally. Lateral to the upper pole on the right was a flat, bean-shaped mass approximately 2 cm. in length, 1 cm. in width and

TABLE III

Calcium Intake and Output

Date	Calcium intake	Calcium output	
		Urine *	Stools †
	mg.	mg.	mg.
Nov. 11	110	153	37.5
" 12	102	64	20.8
" 13	95	140	31.0
Total	307	357	89.3

Total calcium output	446 mg.
Total calcium intake	307 mg.
Negative balance	139 mg.

* Determinations by Miss H. M. Hunt, New England Deaconess Hospital.
† Determinations by Dr. Alexander Marble, Massachusetts General Hospital.

3 mm. in thickness. It was wax-brown in color and, except for its size, resembled a parathyroid. A subtotal hemithyroidectomy was done on the right side, excising the tumor at the lower pole and the mass lateral to the upper pole. At the left lower pole was palpated a nodule apparently arising from the gland itself. This was excised."

During the next few days following the operation, the patient ran a rather stormy course, with persistent vomiting and a rapid pulse. The blood calcium on the day following the operation was 13.8 mg. as compared with 15 mg. on the day before operation. Eight days after operation it was 12.9 mg., the lowest reading. On December 2, fifteen days after operation, it had risen to 14.1 mg. During the postoperative period her condition was not sufficiently stable to carry out studies of calcium balance. By the time her condition had im-

proved to the extent that they could be started, her relatives wished to take her home and accordingly these studies were unfinished. She died at home six weeks later. An autopsy was not permitted.

Microscopic examination (No. 6839) of tissue removed from region of parathyroid showed a mass of epithelial cells occurring in clusters, occasionally showing an alveolar arrangement, and embedded in a relatively dense stroma. The cells were moderate in size, polyhedral, with large hyperchromatic nuceli. The cell membranes were ill-defined, the cytoplasm fairly dense and acidophilic but free from granules. Rare mitoses were present, but no abnormal or multiple mitoses were seen. No tumor giant cells were encountered. Near the periphery of the tissue were lymphatics distended with masses of tumor cells. Blood vessels, however, were not involved, except a few of the larger arteries which showed invasion of tumor cells into their perivascular lymphatics. The alveolar arrangement was discernible even within the lymphatics. No secretion was present, however.

The thyroid itself contained an adenomatous nodule 4 cm. in diameter, which had undergone cystic degeneration. Microscopic examination of the wall of the nodule showed invasion in several places by clusters of epithelial cells similar to those described above. Other portions of thyroid tissue showed marked variation in size of follicles, many of which were distended with a considerable amount of colloid, and scattered foci of isolated follicles of fetal type embedded in a rather myxomatous stroma. At various points near the capsule of the gland invasion by tumor cells similar to those described above was seen.

Microscopic Diagnosis: Adenocarcinoma, metastatic to thyroid and parathyroid, probably originating from the breast.

Discussion

Although we felt that we were dealing with a case of generalized carcinomatosis arising from the carcinoma of the breast removed five years before, we were unable to reconcile the persistent hypercalcemia with this diagnosis. Personal experience and a search of the literature showed no similar finding in bone metastases in carcinoma. In addition there was a tumor in the region of the parathyroid. The flaccid muscles with the attendant weakness, vomiting, polydipsia and polyuria could be explained by the hyper-

calcemia. In view of these considerations, together with the widespread bone changes, a diagnosis of hyperparathyroidism was considered possible and was the basis for operation.

The discovery of the malignant nature of the tumor was discouraging to the hypothesis of hyperparathyroidism. While the possibility of a primary malignant tumor of the parathyroid invading the adjacent thyroid and not yet giving rise to metastases has to be considered, there are several facts which militate very strongly against this. The morphology of the tumor is strikingly suggestive of adenocarcinoma of the breast. So far as we know, there are no malignant tumors of the parathyroid which have produced hypercalcemia. The character of the roentgenograms of the bone lesions is far more suggestive of carcinomatous metastases than it is of decalcification resulting from hyperparathyroidism. Finally, the rarity of malignant tumors possessed of highly specialized specific functional activity must be considered.

Carcinoma of the breast has been reported by Thompson [9] as metastasizing to the parathyroid glands. He also referred to a case reported by Pepere.

This case is presented as a case of marked hypercalcemia. If marked hypercalcemia is always due to hyperfunction of the parathyroids, we were dealing with a case of hyperparathyroidism. If the hypercalcemia was merely an accompaniment of widespread bone metastases, there are no similar cases recorded in the literature. This report should stimulate further estimation of the serum calcium in metastatic bone carcinoma.

Summary

A case with widespread bone changes, hypercalcemia, negative calcium balance and thyroid tumor, is presented. The X-ray appearance of the bones suggested metastatic carcinoma. Examination of parathyroid-like bodies removed at operation showed probable metastatic carcinoma from mammary cancer removed five years before.

REFERENCES

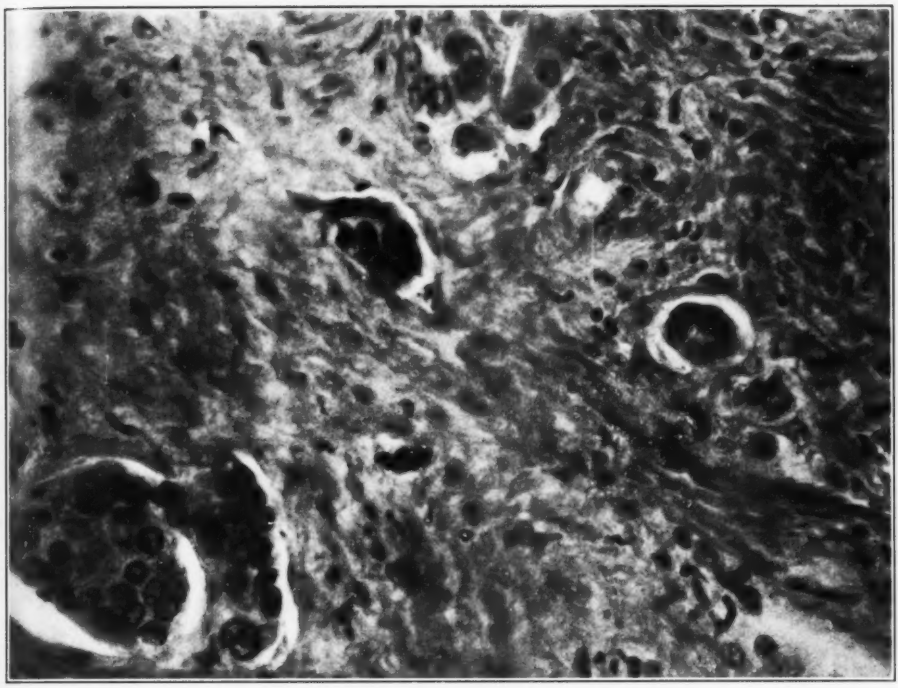
1. Collip, J. B. *J. Biol. Chem.*, 1925, **63**, 395.
2. Richardson, E. P., Aub, J. C., and Bauer, W. *Ann. Surg.*, 1929, **90**, 730.
3. Pemberton, J. de J., and Geddie, K. B. *Ann. Surg.*, 1930, **92**, 202.
4. Wilder, R. M. *Endocrinology*, 1929, **13**, 231.
5. Barr, D. P., and Bulger, H. A. *Am. J. M. Sc.*, 1930, **179**, 449.
6. Hunter, D. *Proc. Roy. Soc. Med.*, 1929, **23**, 227.
7. Albright, F., Bauer, W., Ropes, M., and Aub, J. C. *J. Clin. Investigation*, 1929, **7**, 139.
8. Bauer, W., Albright, F., and Aub, J. C. *J. Clin. Investigation*, 1929, **7**, 75.
9. Thompson, R. L. *J. Med. Res.*, 1911, **24**, 291.

DESCRIPTION OF PLATE

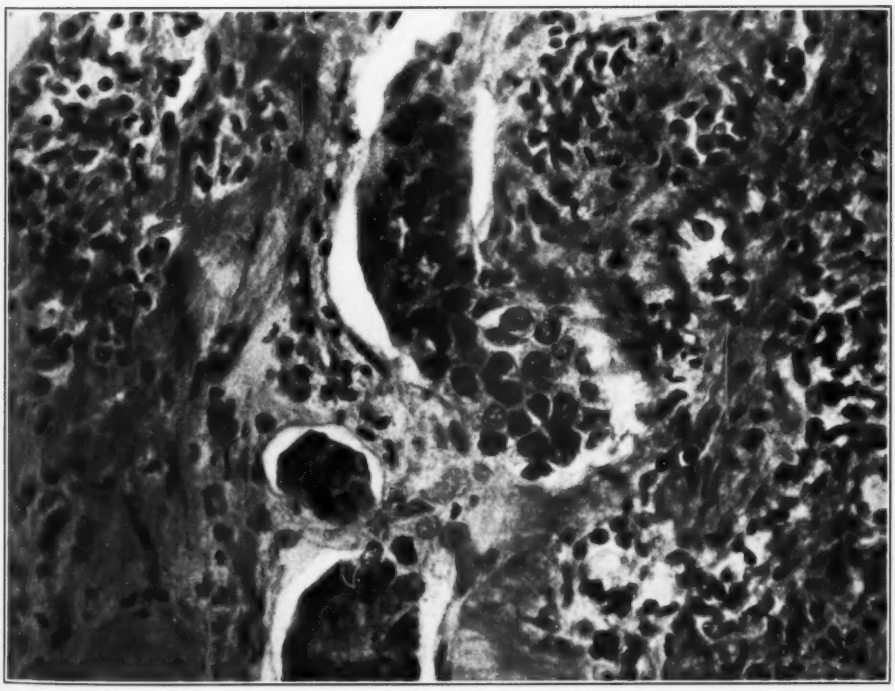
PLATE 81

FIG. 1. Section from mass of tumor tissue from region of parathyroid. Phosphotungstic acid hematoxylin stain. × 400.

FIG. 2. Tumor tissue growing in lymphatic just within capsule of thyroid. Phosphotungstic acid hematoxylin stain. × 400.



1



2